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STUDIES CONCERNING THE RELATIONSHIP BETWEEN PNEUMOCOCCI AND STREPTOCOCCI.

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From time to time evidence has been presented that a close relationship exists between Pneumococcus and Streptococcus. Kruse and Pansini (1), observing the variability of Pneumococcus when grown under different conditions, drew attention to the resemblance between certain races of Pneumococcus and Streptococcus and even suggested the possibility that these bacteria might have their origin in a common, probably saprophytic form of streptococci. Since then a number of observers have brought forth evidence indicating that *Streptococcus hæmolyticus, Streptococcus viridans, Streptococcus mucosus* and Pneumococcus are so closely related that under certain conditions one may change into the other. The evidence for this point of view has recently been greatly supported by the series of publications of Morgenroth and his collaborators and assistants.

Morgenroth, Schnitzer and Berger (2) have reported that by special methods it was possible to transform with great regularity pneumococci into streptococci.

The methods employed were the following: Pneumococci were cultivated in medium containing dead yeast cells or animal charcoal which had absorbed optochin. Under these conditions the pneumococci underwent modifications and were transformed into cocci which possessed properties held to be characteristic for *Streptococcus viridans*. The organisms were now insoluble in solutions of sodium taurocholate, were avirulent for mice, were resistant to the action of optochin and were more sensitive to the action of rivanol than was the original strain. According to these writers, this transformation of pneumococci into streptococci did not occur suddenly but in undergoing the transformation the bacteria passed through several stages or modifications.

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Bacteria in the first stage of the process were described as Modification A. While the bacteria in this stage retained most of the characteristic properties of pneumococci, they had become more resistant to the action of optochin. When blood agar plates were made from the Modification A culture, after growing in optochin, it was found that the colonies no longer resembled those of the original culture but now resembled the colonies of *Streptococcus viridans*. Microscopically the organisms appeared as long chains of round individuals; they were bile-insoluble and were only killed by very high concentrations of optochin. This form was called Modification B and was considered to be identical with *Streptococcus viridans*. Upon further growth in artificial medium, or in the animal, and occasionally upon growth in optochin-containing medium, the third form, Modification C, was found to arise. These organisms corresponded in their properties to *Streptococcus hæmolyticus*, that is, they grew on blood agar with more or less hemolysis, were bile-insoluble and optochin-fast. When injected into the subcutaneous tissue of the abdomen of the mouse phlegmons formed. Moreover, these organisms showed the sensitiveness to the action of rivanol which is characteristic for *Streptococcus hæmolyticus*. Occasionally the transition of Modification A into Modification C occurred without passing through the intermediate stage of Modification B.

These observations concerning the changes undergone by pneumococci and streptococci are of great importance not only in their bearing on the classification of these microorganisms, but also in their relation to the pathogenesis of human infection. It has seemed of importance, therefore, to repeat those experiments and to attempt to interpret them in the light of studies made by the writer (3, 4) and others concerning the variations in the characteristics of Pneumococcus which occur under a number of conditions.

It has been found by a number of observers that when pneumococci are grown in various unusual ways, such as in immune serum or in bile, atypical colonies may appear. These have been called R, or rough, colonies in contrast with the S, or smooth, colonies which are the typical pneumococcus colonies. The bacteria of the R colonies are avirulent for mice, have no capsule, do not produce the so called pneumococcus soluble substance, are somewhat resistant to the action of bile and have lost their type specificity. They have been found to retain their properties on further cultivation and even after repeated passage through mice. Blake and Trask (5) have described a third type of colony, probably made up of intermediate forms, the bacteria of which may revert to the typical virulent pneumococci on passage

through mice. The R bacteria although to some degree resembling streptococci of the *viridans* variety have nevertheless been considered by the writer and by most of the workers on this problem to be modified pneumococci.

Methods.

In studying the modifications undergone by pneumococci when exposed to the action of optochin or optochin absorbed on yeast or on animal charcoal, the following methods were employed.

A strain of Pneumococcus derived from a single cell of a Type I stock culture was employed. 0.05 cc. of a 1–10 dilution of an 18 hour broth culture of this organism was inoculated into serum bouillon containing varying concentrations of optochin hydrochloride (ethyl hydrocupreine hydrochloride, Zimmer and Co.) or into serum broth containing varying concentrations of optochin absorbed on yeast. These cultures were incubated from 18 to 20 hours and plated on blood agar to detect colony variations. Yeast-absorbed optochin was prepared as follows: A 10 per cent suspension of dried yeast (Fleischmann) in distilled water was made and this was sterilized by heat and then centrifuged. The supernatant fluid was replaced with a 1–10,000 solution of optochin hydrochloride in distilled water and the mixture incubated ½ hour. The suspension was again centrifuged, the supernatant fluid discarded and replaced by twice the amount of 10 per cent serum broth.

For testing bile solubility, 0.5 cc. of sterile, undiluted ox bile was added to 0.5 cc. of a broth suspension of bacteria and incubated for 2 hours at 37°C.

Experimental.

An 18 hour broth culture of Pneumococcus was inoculated into broth containing a suspension of yeast-absorbed optochin as described above and incubated 20 hours. After plating on blood agar only one kind of colony appeared and this resembled exactly the S form of colony which has been described in a previous paper (3). The original culture was now passed two more times through the yeast-absorbed optochin medium. From this third culture, plates were made and now a number of small, thin, rough surfaced colonies, like those which have previously been described as R forms, appeared. A study was next made of the changes undergone by pneumococci when grown in broth containing various amounts of optochin without any yeast being present, and also when grown in suspensions of yeast containing no optochin. The culture was grown repeatedly in serum broth containing optochin hydrochloride in concentration of 1–100,000. At the ninth transfer the concentration was increased to 1–50,000 and at the thirtieth transfer, to 1–20,000. Upon plating the culture at intervals during the repeated transfers, no small R colonies appeared. This last experiment was repeated, substituting plain broth for serum broth. Three transfers were made in this medium con-

taining optochin hydrochloride in a concentration of 1–600,000. When this third culture was plated on blood agar, three kinds of colonies were found, S colonies, R colonies and colonies of an intermediate form like those described by Blake and Trask.

The culture was also grown repeatedly in a 10 per cent suspension of heat-killed yeast in serum broth, no optochin being present. Transfers were made at 6 hour intervals on account of the tendency of the organisms to die in th's medium. After the fourth transfer in this medium, plating on blood agar showed a great preponderance of colonies of the R form over those of the S forms.

These experiments, which have been repeated a number of times, indicate that in our hands the result of growing typical pneumococci in media containing yeast which has absorbed optochin, or even in media containing optochin alone or yeast alone, is the appearance of modified pneumococci which form colonies identical with those described by a number of writers as R colonies. From the description of the colonies of Modification B as given by Morgenroth and his associates, it seems probable that the bacteria of Modification B are identical with those we and others have described as pneumococci of the R colonies.

Morgenroth and his collaborators consider these to be true streptococci of the *viridans* type. We have called them modified pneumococci. It is true, as Morgenroth and his collaborators state, that these colonies resemble those of *Streptococcus viridans*. Moreover, the bacteria from these colonies have little virulence for mice and they are resistant to optochin and do not dissolve as readily as do typical pneumococci in solutions of bile. The writers we have mentioned state that these organisms are insoluble in solutions of sodium taurocholate and they lay stress on this property as indicating their identity with the bacteria of the *Streptococcus viridans* group. In carrying out the bile test, however, Morgenroth and his associates have employed the method of Levy (6), adding a 2.5 per cent solution of sodium taurocholate to the broth culture. We have modified this technic, employing not only a 2.5 per cent solution of this substance, but also solutions of a higher concentration. The effect of ox bile itself on these organisms has also been tested. Many tests of R cultures have been made, and at the same time tests have been made of twelve different cultures from various sources which had previously been identified by another worker as cultures of *Streptococcus viridans*.

The latter cultures were all found to be insoluble in ox bile and also in sodium taurocholate solutions, even when this was highly concentrated. On the other hand, when the bacteria of the R colonies or what Morgenroth and his associates have called Modification B bacteria, were tested, it has been found that although they are resistant to the action of sodium taurocholate in 2.5 per cent solution, they are dissolved if higher concentrations of this salt be employed. Moreover, if the bacteria be suspended in salt solution instead of in broth, solution occurs even in 2.5 per cent solutions of sodium taurocholate. Finally, if ox bile be employed, these organisms all dissolve, although more slowly than usually occurs with typical pneumococci. Our experiments, therefore, indicate that these modified bacteria are not, properly speaking, bile-insoluble; they possess increased resistance to the action of bile. They, therefore, differ in this respect from bacteria of the *Streptococcus viridans* group which are truly bile-insoluble. They also differ from *Streptococcus viridans* in certain other properties. We have previously noted the readiness with which pneumococci undergo autolysis when they are suspended in saline solutions and kept at 37°C. This readiness to undergo autolysis is seen in pneumococci of all types and is especially well marked in bacteria from the R colonies. On the other hand, streptococci of all varieties undergo autolysis very slowly.

Saline suspensions of six strains each of the S and R forms of pneumococci and of ten strains of typical *Streptococcus viridans* were made and placed in the incubator at 37°C. for 48 hours. At the end of this time the tubes were centrifuged and stained films were prepared from the sediment of each tube. It was found that well formed, Gram-positive cocci were still present in all the tubes containing streptococci, while in the tubes containing the S and R forms of pneumococci no intact cells remained.

We have reported elsewhere (7) that freezing and thawing easily causes disruption of the cell bodies of pneumococci, while this treatment has no effect on streptococci. Pneumococci of the R form obtained by growth in serum and in bile and also modified pneumococci obtained by growth in media containing optochin absorbed on yeast have been tested by these methods. In every instance all these

bacteria have been found to be readily disintegrated by the action of freezing and thawing.

Finally, the immunological properties of atypical pneumococci derived from type-specific strains by growth in broth containing yeast-absorbed optochin were compared with the immunological properties of typical streptococci of the *viridans* group.

We have previously reported observations concerning the immunological reactions of the R forms of pneumococci (4). It was there shown that sera prepared with each of these strains agglutinated all other R forms, no matter how obtained or from what type of Pneumococcus. These sera also caused precipitation of the protein derived from all kinds of pneumococci. The interpretation placed on these experiments was that the R forms of pneumococci were pneumococci which had lost the property of producing the pneumococcus type-specific carbohydrate substance.

Immune sera were now prepared by injecting rabbits with cultures of *Streptococcus viridans*. Six different strains were employed, an immune serum being produced against each one of these strains. These special strains were selected because they grew diffusely in broth. Each of these sera was found to be specific for the homologous organism, causing agglutination usually in dilutions as high as 1–320. In the concentrated serum there occurred some crossing or non-specific agglutination of the other *Streptococcus viridans* strains. All but one of the sera also agglutinated suspensions of R pneumococci but the agglutinating power for these organisms was not as great as that of the serum prepared by the injection of R pneumococci. By absorption of the agglutinins for the R strains by R organisms the agglutinins of the antistreptococcus sera for heterologous *Streptococcus viridans* strains were removed and the absorbed sera were now race-specific and agglutinated only the homologous race of *Streptococcus viridans*. Anti-R pneumococcus sera, on the other hand, did not agglutinate any of the six strains of *Streptococcus viridans* studied.

The following interpretation of these results, in the light of the work of Heidelberger and Avery (8) seems justifiable. Lancefield (9) has previously shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of Pneumococcus. Individual strains of *Streptococcus viridans* also possess a substance

comparable to that of the soluble specific substance of Pneumococcus and this endows them with the power of reacting specifically with their homologous serum. To what degree a single specific substance is common to a number of *Streptococcus viridans* strains, only further work will show. The protein of all strains being alike and being common to all pneumococci, the serum produced by each *Streptococcus viridans* strain agglutinates all R pneumococci. The anti-R serum, however, does not agglutinate the *Streptococcus viridans* strain because each of these strains has a specific reacting substance, which masks or prevents the non-specific antiprotein reaction. This is analogous to the immunological phenomena exhibited by the specific pneumococci. These organisms, although they contain the non-specific pneumococcus protein, are not agglutinated by anti-R serum or by anti pneumococcus-protein serum. The explanation offered for the immunological phenomena exhibited by strains of the *Streptococcus viridans* group also receives support from the work of Zinsser on hemolytic streptococci (10).

It seems from all of this work that the atypical forms of bacteria obtained by growing type-specific strains of pneumococci in media containing serum or bile or by growing them in dilutions of optochin, or in yeast-absorbed optochin according to the method of Morgenroth, are not strains of *Streptococcus viridans* but are in fact degraded forms of pneumococci.

The reasons we have adduced for this are as follows:

1. Degraded forms of pneumococci are not bile-insoluble, as Morgenroth and his associates have stated, although they are more resistant to bile than are typical pneumococci.

2. Degraded forms of pneumococci behave like typical pneumococci and not like streptococci in the readiness with which they undergo autolysis and in the ease with which the cell bodies are broken down by freezing and thawing.

3. Immune sera produced by the injection of animals with degraded strains of pneumococci behave toward typical strains of *Streptococcus viridans* just as they do toward specific strains of Pneumococcus. Typical strains of *Streptococcus viridans* possess a type-specific immunological mechanism just as do typical pneumococci. The species-specific immunological mechanism possessed by *Streptococcus viridans*

is identical with that of Pneumococcus.   To this extent Pneumococcus and *Streptococcus viridans* are closely related.   Whether a typical type-specific strain of Pneumococcus, however, is ever changed into one of *Streptococcus viridans* any more than a Pneumococcus of Type I is ever changed into one of Type II or of Type III only further work will show.   The evidence for this at the present time is not convincing.

Experiments with Hemolytic Streptococci.

Morgenroth and his associates (11) also report that they have been able, by special methods, to convert strains of *Streptococcus hæmolyticus* into those of *Streptococcus viridans*.   This change was easily induced by cultivating hemolytic streptococci in serum broth containing rivanol bound to erythrocytes, *"haut-pulver,"* animal charcoal or yeast. Rivanol solutions alone in serum broth were not efficacious.   Rivanol (2-ethoxy-6, 9-diaminoacridium hydrochloride) is a salt which is said to have a specific germicidal action for *Streptococcus hæmolyticus*.

Other investigators have also observed hemolytic streptococci change into non-hemolytic forms.   Valentine and Krumwiede (12) on one occasion observed the spontaneous appearance of occasional green colonies on a blood agar plate made from a culture of *Streptococcus hæmolyticus* (Strain 32, Dochez, Avery and Lancefield (13)). Bacteria from both kinds of colonies were agglutinable in the original strain antiserum and each absorbed the common antibody.   Lehmann (14) obtained green-producing colonies from 3 out of 87 strains of hemolytic streptococci which had been cultivated in milk media.

On account of the bearing which these observations concerning streptococci have on the preceding experiments, it seemed of interest to repeat the experiments of Morgenroth and his associates.

Methods.

A 10 per cent suspension of dry yeast in distilled water was sterilized and centrifuged as described previously.   The supernatant fluid was replaced with a 1–1,000 solution of rivanol (Metz) in distilled water and incubated for 1 hour at 37°C.   *After centrifuging, the sediment was washed three times with distilled* water and made up to the original volume.   Varying dilutions of this suspension were made in 10 per cent serum broth.   0.05 cc. of a 1–10 dilution of a broth culture of *Streptococcus hæmolyticus* was inoculated into 2 cc. of the respective dilutions of yeast-absorbed rivanol and incubated at 37°C. for 20 hours.

In order to obtain pure line cultures, in certain of the experiments single chains of 3 or 4 cocci were isolated according to the technic described in a previous paper (3). Single cocci are difficult to isolate on account of their small size and resemblance to particulate matter in the agar.

Experimental.

Twenty-six strains of hemolytic streptococci, including stock cultures and freshly isolated strains, were tested. Bacteria were inoculated in serum broth containing rivanol alone and in serum broth containing yeast alone, but in most instances in dilutions of rivanol-yeast suspensions varying in strength from 1–4 to 1–8,000. The organisms tended to die in the stronger concentrations and for prolonged growth required alternate transfers into plain serum broth. Cultures could be transferred indefinitely in dilutions weaker than 1–1,000.

After each transfer the organisms were plated on blood agar, incubated at 37°C. and the plates examined for the presence of atypical colonies. Approximately, 400 transfers were made in the various media and only on the plates made from one of the strains (Streptococcus 267) were atypical colonies seen. The variant colonies which occasionally appeared on the blood agar plates among the hemolytic colonies of this strain were anhemolytic, and the organisms derived therefrom were avirulent and bred true on further culture. However, atypical colonies appeared a number of times spontaneously when this culture, without any previous treatment with yeast-absorbed rivanol, was plated on blood agar.

To eliminate the possibility of the variant being a contaminant, 20 single short chains were isolated from the original culture and treated with yeast-absorbed rivanol in dilutions varying from 1–1,000 to 1–8,000. Under these conditions no variant colonies appeared. This experiment does not entirely prove that the variants were contaminants but this explanation seems not improbable.

Two of the original cultures, S3 and S23, were transferred 70 times on plain agar before treatment with yeast-absorbed rivanol. In spite of this preliminary treatment no variants ever appeared.

SUMMARY AND CONCLUSIONS.

Morgenroth and his collaborators grew pneumococci in a medium containing optochin and derived variant forms of bacteria therefrom which were considered to be streptococci of the *viridans* group. We have repeated these experiments and have also derived variant forms. These atypical races, however, we have found to be identical with the R form of pneumococci which have been obtained by various other methods. That these R cultures are still pneumococci and do not belong in the *Streptococcus viridans* group is supported by the following observations:

1. These strains are not bile-insoluble, but are more resistant to the lytic action of this agent than are type-specific pneumococci.

2. R strains behave like pneumococci and not like streptococci in the readiness with which cultures spontaneously autolyze and saline suspensions disintegrate during freezing and thawing.

3. Immunological reactions of the variant pneumococci derived by Morgenroth's method are identical with the immunological reactions of R forms of pneumococci derived by various other means.

The observations of Morgenroth and his associates in regard to the transformation of *Streptococcus hæmolyticus* into *Streptococcus viridans* by treatment with rivanol could not be repeated in this laboratory. No explanation is at hand for our failure to produce the change. It is of course possible that none of the twenty-six strains tested had suitable tendencies to variation or that the technic or reagents employed varied from those of Morgenroth.

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EFFECT OF REPEATED FREEZING (−185°C.) AND THAWING ON COLON BACILLI, VIRUS III, VACCINE VIRUS, HERPES VIRUS, BACTERIOPHAGE, COMPLEMENT, AND TRYPSIN.

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The effect of low temperatures on bacteria, viruses, ferments, cells, and the bacteriophage has been observed and reported. The effect of repeated freezing and thawing on some of them has also been studied. The temperatures used, however, as well as the number of freezings and thawings employed, varied greatly, and in many instances the effect on only one or two agents was studied by the same worker. Nevertheless some observers have stated that by means of repeated freezing and thawing it is possible to determine whether certain of these agents are living or dead. In view of the inadequacy of the knowledge concerning this subject it seemed desirable to study further the effect of repeated freezing (−185°C.) and thawing on vaccine virus, Virus III, herpes virus, and the bacteriophage as compared with the effect on colon bacilli, complement, and trypsin. The results of the investigation are reported in this paper.

Macfadyen (1–3) exposed vegetative and spore forms of bacteria, seed, and Buchner's zymase to the temperatures of liquid air (−185°C.) and liquid hydrogen (−252°C.) for 20 hours and found that the bacteria grew, that the seed germinated, and that the zymase was still active. No determinations of the number or amount of the active agents, however, were made before or after the exposures.

White's (4) report concerning the results obtained by exposing bacteria to the temperature of liquid air for a period of 2 hours shows that 50 per cent of the hardier bacteria survived while 98 per cent of the less resistant varieties were killed. He also states that vaccine virus was active after an exposure for 15 minutes to the temperature of liquid air. No determinations of the amount of active virus were made, however, before or after the exposure.

Barratt (5) found that rabies virus was active after an exposure for 3 months

to the temperature of liquid air. The same virus, however, was not active after treatment for 45 minutes in a disintegrating machine kept at a temperature of $-185°C$.

Salvin-Moore and Barratt (6) exposed cells of a graftable mouse cancer to the temperature of liquid air for 30 minutes and found that mice inoculated with the treated cells developed cancers. The observers assume that the cells were killed by the treatment while the cancer virus survived.

Gaylord (7) found that cells of a transplantable mouse cancer, although injured, were able, after exposure for 80 minutes to the temperature of liquid air, to produce tumors when injected into mice. On the other hand, he found that freezing, by means of liquid air, killed embryonic tissue. He also observed that trypanosomes were able to resist a temperature of $-185°C$. for a period of 20 minutes but not for a period of 40 minutes.

Stockman and Minett (8) state that the virus of foot and mouth disease is active after repeated freezing and thawing. Ammonia brine was used for freezing.

Sanderson (9), using two strains of bacteriophage, one active for staphylococci, the other lytic for colon bacilli, found that the titer of the phages was not influenced by freezing and thawing 20 successive times. Solid CO_2, approximately $-78°C$., was used for freezing. The results of his experiments lead him to believe that the bacteriophage is something other than a viable organism.

D'Hérelle (10), working with two strains of bacteriophage, one active for dysentery bacilli, the other lytic for staphylococci, found that the phage in filtrates less than 17 days old resisted at least 3 exposures to the temperature of liquid air for periods of 10 minutes each, while the phage in older filtrates was not active after 1 to 3 such exposures.

Methods and Materials.

Freezing and Thawing.—The low temperature ($-185°C$.) used in the experiments to be reported was obtained by means of commercial liquid air which was transported from the plant to the laboratory in Dewar flasks. Desired amounts of the air were transferred to deep Dewar beakers where small amounts of the substances to be frozen, enclosed in Noguchi tubes, were completely immersed for several minutes. After the substances had been completely frozen they were quickly thawed in tap water (16–18°C.). This procedure was repeated as often as desired. Then the treated substances, as well as the controls, were tested and titrated by standard methods.

B. coli.—Colon bacilli, 24 hours old, were washed off agar slants and suspended as desired in water, broth, physiological salt solution, or Locke's solution.

Vaccine Virus.—Testicles, into which vaccine virus had been injected 4 days previously, were removed from the rabbit and ground up with sand. After the addition of 20 cc. of Locke's solution, the emulsion was centrifuged, and the supernatant fluid was used as a stock virus. A fresh stock was made for each experiment.

Virus III.—Virus III (11), indigenous to rabbits, was prepared for each experiment in a manner similar to that described for vaccine virus.

Herpes Virus.—Herpes virus was injected into the brain* of a young rabbit. The animal usually died 5 days later. From the brain, removed immediately after death, a 1–10 emulsion was made in Locke's solution and centrifuged. The supernatant fluid was used as a stock virus. Fresh stock was made for each experiment.

Bacteriophage.—A bacteriophage lytic for *B. coli* was prepared in the usual manner in buffered broth with colon bacilli. The resistent bacteria were removed by filtration and the filtrate was used as a stock phage. The filtrates used in the experiments were 19 to 90 days old and the lytic principle in them was usually active in a dilution 10^{-10}. Titrations for the activity of the phage were made in the usual manner by means of dilution with broth in which young colon bacilli were suspended. After the different dilutions were incubated for 48 hours, the cloudy tubes were heated at 60°C. for 30 minutes, and then 0.5 cc. from each tube was tested for the presence of phage. In this way the last tube in which phage was present could be accurately determined.

Complement.—Complement in fresh guinea serum was used. Dilutions of the serum were made by means of physiological salt solution. Activity of the complement was tested in the usual way by dilution or by the determination of the length of time required by the complement to hemolyse a known amount of red blood cells. Care was taken not to inactivate any of the complement by shaking.

Trypsin.—Trypsin, in a buffered solution of pH 7, was supplied by Dr. Northrop. Determinations of the ferment's activity before and after freezing and thawing were made by Dr. Northrop. Details of the technic can be obtained from his papers (12). Care was taken to inactivate none of the ferment by shaking.

EXPERIMENTAL.

All experiments made to determine the effect of freezing (-185°C.) and thawing on colon bacilli, Virus III, vaccine virus, bacteriophage, complement, and trypsin were repeated several times, and many of them were performed simultaneously. For convenience, however, the results of the experiments will be reported separately.

Colon Bacilli.

It is a well established fact that many bacteria are killed by repeated freezing and thawing, even when a temperature only a few degrees below 0°C. is employed. Therefore colon bacilli were in-

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* All operations were performed under ether anesthesia.

cluded as a control in the list of active agents to be exposed repeatedly to the temperature of liquid air, and the following experiment is illustrative of the results obtained with these organisms.

Colon bacilli, washed from an agar slant and suspended in broth, were frozen and thawed 12 successive times. Before freezing there were 180,000,000 viable organisms per cc. After freezing and thawing, however, only 40,000 organisms per cc. remained viable.

When it had been shown by the experiment above that colon bacilli are killed by repeated exposure to the temperature of liquid air, the following experiment was performed to determine what influence the number of bacteria per cc. and the type of suspending fluid have on the percentage of organisms killed by repeated freezing and thawing.

TABLE I.

Summary of Experiment Showing the Effect of Dilution and Diluent on the Percentage of Colon Bacilli Killed by Freezing and Thawing 4 Successive Times.

Diluent	Dilution	No bacteria per cc before freezing	No bacteria per cc. after 4 freezings and thawings
Locke's solution.....................	1-10	18,400,000	27,000
" "	1-1,000	380,000	20
Broth	1-10	44,000,000	13,200,000
"	1-1,000	600,000	188,000

Colon bacilli, removed from an agar slant, were suspended in sterile distilled water. 0.5 cc. of the suspension was put in 4.5 cc. of buffered broth and Locke's solution respectively. Further dilutions, 1-1,000, were made with each diluent, and two of the suspension, 1-10 and 1-1,000, in each series were frozen and thawed 4 times. The number of viable organisms per cc. was determined before and after freezing. The results of the experiment are summarized in Table I.

It is obvious from the results shown in Table I that the colon bacilli were more susceptible to repeated freezing and thawing when Locke's solution instead of broth was employed as a suspending fluid. It is also evident that approximately the same percentage of bacteria was killed in the different suspensions made by means of broth. On the other hand, a greater percentage of bacteria was killed in the 1-1,000 dilution made with Locke's solution than in the 1-10 dilution made with the same fluid.

Virus III.

The effect of repeated freezing and thawing on Virus III was determined by means of the following experiments.

1 cc. of a fresh stock emulsion containing active Virus III was added to 9 cc. of Locke's solution. One half of the emulsion was used as a control; the other was frozen and thawed 22 successive times. From the second half samples were removed for tests after the 2nd, 12th, and 22nd freezings. The control emulsion and the samples of the treated one were tested for the presence of active Virus III by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are shown in Table II.

TABLE II.

Summary of the Results Obtained by Repeated Freezing and Thawing of Virus III.

No. of freezings and thawings	Dilutions		
	1–10	1–100	1–1,000
Control (unfrozen)	++	+±	+
2	++	+	±
12	−	−	~
22	−	−	~

+'s indicate the presence of a virus reaction at the sites of inoculation. − indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

The experiment indicates clearly that Virus III is easily killed or inactivated by repeated freezing and thawing in spite of the high protein content of the emulsion containing the virus.

Vaccine Virus.

Vaccine virus in a stock testicular emulsion, diluted 1–10, 1–100, and 1–1,000, showed no appreciable decrease in its titer after 12 successive freezings and thawings. Finally, however, it was possible as will be shown in the following experiment, to decrease the titer of the virus by repeated freezing and thawing.

1 cc. of a stock testicular emulsion containing active vaccine virus was diluted 1–10, 1–100, and 1–1,000 by means of Locke's solution. One half of the 1–1,000 dilution was used as a control; the other half was frozen and thawed 34 successive

times. Samples of the latter half were removed for tests after the 24th and 34th freezings. The control emulsion and the samples of the treated one were tested for the presence of active vaccine virus by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are summarized in Table III.

From the results of the experiment just described it is evident that a certain percentage of the vaccine virus, diluted 1–1,000 with Locke's solution, was inactive after 34 successive freezings and thawings. This virus, however, proved to be more resistant than any other of the active agents tested.

TABLE III.

Summary of the Results Obtained by Repeated Freezing and Thawing of Vaccine Virus.

No. of freezings and thawings	Dilutions		
	1–1,000	1–10,000	1–100,000
Control (unfrozen)	+++	++	+
24	++	+	±
34	++	+	–

+'s indicate the presence of a virus reaction at the sites of inoculation. – indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

Herpes Virus.

The activity of herpes virus in a fresh stock emulsion was not appreciably decreased by 12 successive freezings and thawings. The following experiment, however, shows that the virus is susceptible to such treatment under certain conditions.

0.5 cc. of a fresh stock brain emulsion containing active herpes virus was diluted 1–20 by means of Locke's solution. One portion of the diluted emulsion was used as a control; another portion was frozen and thawed 24 times. The two portions were then tested for the presence of active virus by means of intracerebral inoculations in young rabbits. The results of the tests are summarized in Table IV.

The experiment demonstrates that herpes virus, provided the brain emulsion containing it has been sufficiently diluted by means of Locke's solution, is killed or inactivated by repeated freezing and thawing.

Bacteriophage.

Early in the work with a bacteriophage lytic for colon bacilli it became evident that repeated freezing and thawing inactivated at least 99 per cent of the phage. Details of these experiments will be omitted since results similar to the ones obtained in them will be

TABLE IV.

Summary of Results Obtained by Repeated Freezing and Thawing of Herpes Virus.

No. of freezings and thawings	Dilutions		
	1–20	1–40	1–80
Control (unfrozen)	+	+	+
24	−	−	−

+ indicates death of the animal within 7 days after intracerebral inoculation of 0.25 cc. of the emulsion diluted as noted. − indicates that the animal was alive 4 weeks after inoculation. Dilutions are in terms of the stock emulsion.

TABLE V.

Summary of Experiment Showing the Effect That the Type of Diluent Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage after 12 freezings and thawings
Broth (unfrozen control)..................	1–10,000	10^{-11}
"	1–10,000	10^{-7}
Distilled water.........................	1–10,000	10^{-6}
Locke's solution........................	1–10,000	10^{-5}
Salt "	1–10,000	Completely inactivated

Dilutions are in terms of the stock filtrate.

shown in other experiments performed to ascertain what influence the degree of dilution or the type of diluent has on the percentage of phage inactivated by repeated freezing and thawing. The effect that the type of diluent has on the percentage of phage inactivated under these conditions was investigated first in the following manner.

Portions of the stock filtrate containing the active lytic substance were diluted 1–10,000 respectively by means of buffered broth, Locke's solution, distilled

water, and physiological salt solution. The pH of the first two diluents was 7.6, while that of the last two was between 7 and 6. Small amounts of the diluted filtrate were frozen and thawed 12 successive times. Then tests were made in the usual way to determine the titer of active phage in the control as well as in each treated specimen. The results of the titrations are shown in Table V.

The experiment affords conclusive evidence (1) that phage is inactivated by repeated freezing and thawing, and (2) that the type

TABLE VI.

Summary of Experiment Showing the Effect That Dilution Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage before and after 8 freezings and thawings
Broth (control)................................	1–10	10^{-11}
" (frozen)................................	1–10	10^{-9}
" "	1–1,000	10^{-9}
" (control)................................	1–1,000	10^{-11}
Distilled water (control)........................	1–10	10^{-11}
" " (frozen)........................	1–10	10^{-8}
" " "	1–1,000	10^{-5}
" " (control)........................	1–1,000	10^{-10}
Locke's solution (control)........................	1–10	10^{-11}
" " (frozen)........................	1–10	10^{-10}
" " "	1–1,000	10^{-7}
" " (control)........................	1–1,000	10^{-11}
Salt solution (control)...........................	1–10	10^{-11}
" " (frozen...........................	1–10	10^{-9}
" " "	1–1,000	10^{-5}
" " (control)...........................	1–1,000	10^{-11}

Dilutions are in terms of the stock filtrate.

of diluent influences the percentage of phage inactivated in this manner.

After it had been determined that the type of diluent does affect the percentage of phage inactivated by repeated freezing and thawing, the following experiment was performed to ascertain what effect the degree of dilution has on the percentage of phage inactivated by such treatment.

Portions of the stock filtrate containing phage were diluted 1–10 and 1–1,000 by means of each of the following diluents: broth, distilled water, Locke's solution, and physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 8 times. Tests were then made in the usual way to determine the titer of active phage in each specimen. In these titrations pipettes were changed after every third tube. Consequently the titer of the phage in each series may be slightly higher than it would have been had a clean pipette been used after each tube. *Since the same technic was used throughout the experiment, the results of the different titrations summarized in Table VI are comparable.*

The results of the experiment indicate that an increase in the dilution of the stock filtrate by means of broth and distilled water

TABLE VII.

Summary of Experiment Showing the Effect That Freezing and Thawing Have on Complement in Diluted Serum.

Specimens of complement	Titration by dilution				Time required for complete hemolysis[*]
	1–50	1–100	1–200	1–400	
1–50 (control)	++	+	±	−	3 min.
1–50 (frozen and thawed).............	+	±	−	−	7 "
1–100 (control).......................		+	±	−	4 "
1–100 (frozen and thawed)...........		−	−	−	No hemolysis

[*] Dr. Landsteiner was kind enough to make this test.

+'s indicate hemolysis.   − indicates no hemolysis.

does not at the same time lead to an increase in the percentage of phage inactivated by repeated freezing and thawing. On the other hand, an increase in the dilution accomplished by means of physiological salt solution and Locke's solution does lead to an increase in the percentage of phage inactivated, as evidenced by a greater percentage of phage being inactivated in dilutions of 1–1,000 than in dilutions of 1–10. These findings are similar to the ones shown in Table I concerning the effect of dilution on the percentage of bacteria killed by repeated freezing and thawing.

Complement.

The titer of complement in undiluted serum and in serum diluted 1–10 is not appreciably decreased by 12 successive freezings and

thawings. When this fact became evident, a study concerning the effect of freezing and thawing on complement in highly diluted serum was made in the following manner.

Portions of fresh guinea pig serum were diluted 1–50 and 1–100 by means of physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 12 times. Then tests for the presence of active complement in each of the control and treated specimens were made by means of dilution and also by the determination of the time necessary for given amounts of the sera to completely hemolyse given amounts of red blood cells in the presence of a great excess of amboceptor. Throughout the experiment, the results of which are shown in Table VII, care was taken not to inactivate the complement by shaking.

From the results of the experiment it may be concluded that complement in highly diluted serum is inactivated by repeated freezing and thawing.

Trypsin.

The effect of freezing and thawing on trypsin was studied in the following manner.

Portions of a 5 per cent stock solution of trypsin were diluted 1–10 and 1–500 by means of a buffered solution having a pH of 7. Half of each specimen was used as a control, the other half was frozen and thawed 12 times. Care was taken to inactivate none of the ferment by shaking. After the amounts of active trypsin in the control and treated specimens were determined[1] and compared, it was found that 70 per cent of the trypsin was inactivated in the specimen diluted 1–500, while only 17 per cent was inactivated in the one diluted 1–10.

The results of the experiment above afford conclusive evidence that trypsin is inactivated by repeated freezing and thawing and under these conditions a greater percentage is inactivated in a dilute than in a concentrated solution.

DISCUSSION.

The numerous discussions concerning the nature of the bacteriophage have led many investigators to question more closely the living nature of some of the so called filterable viruses. Many tests have

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[1] Dr. Northrop supplied the trypsin and determined the amount of active trypsin in the control and treated specimens.

been devised to act as criteria for the presence of life, but so far no one of them has been found satisfactory. Recently Sanderson (9), using a temperature of −78°C., found no diminution in the titer of two strains of phage subjected to 20 successive freezings and thawings. Since bacteria and cells are killed by repeated freezing and thawing he concludes that the bacteriophage must be something other than a living organism. In the experiments reported in the present paper a number of active agents, some undoubtedly living, others equally unquestionably not living, and still others of a doubtful nature, were subjected to repeated freezing (−185°C.) and thawing. By these tests it has been possible to determine that mere destruction or inactivation of a substance cannot be accepted as proof that it existed in a living state.

CONCLUSIONS.

Colon bacilli, Virus III, vaccine virus, herpes virus, bacteriophage, complement, and trypsin are either killed or inactivated by repeated freezing (−185°C.) and thawing. As might be expected, some of the agents are more resistant than others.

Hence it may be concluded that destruction or inactivation of an active agent by repeated freezing (−185°C.) and thawing is not proof that it was possessed of life.

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THE RELATIVE REACTION WITHIN LIVING MAM-
MALIAN TISSUES.

VIII. On the Course of the Tissue Acidosis Secondary to
Blood Acidosis Induced with Hydrochloric Acid.

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In a previous paper (1) the fact has been demonstrated that the reaction of certain tissues can be altered by injecting into the blood amounts of acid or alkali that are compatible with life. Rats vitally stained with a phthalein and receiving dilute hydrochloric acid survive despite marked changes in the color of the connective tissue, tendons, and cartilage. The abnormality in pH thus attested persists for a surprisingly long period. It has seemed possible, by repeating the experiments in larger animals, to learn something of the general relation existing between the reaction of the blood and tissues. Such experiments, upon rabbits, form the main subject of the present paper. But before they are taken up it will be well to give in detail a few observations on the effect upon rats of maximum quantities of hydrochloric acid.

Preliminary Experiments on Rats.

Experiment I.—At 9.20 a.m. a male rat of 123 gm., shaved over the body 24 hours previously, was given an intraperitoneal injection of 1.85 cc. of 4 per cent phenol red isotonic with the blood, but at pH 5.6. The animal was then etherized, a cannula connected with a jugular vein, the body immersed in a warm bath of oil, and, at 10.10 a.m., N/6.35 HCl was run into the circulation. The apparatus and general technic were those employed in a previous paper (2). The hue of the rat to begin with was deeper than *jasper red* (Ridgway) and no change was observed until after 3 cc. of the acid had been given, when the shaved surface appeared somewhat yellower. When 5 cc. was in, at 10.37 a.m., it had become *coral red*. The breathing was exaggerated. By 11.05 a.m. 9 cc. had been injected and the animal was slightly lighter than *rufous*. Urine had been repeatedly voided in

large amount, at first red, then orange-red.   At 11.10, with 9.5 cc. in, a movement of the animal during the course of the stormy respirations loosened the attachment of the cannula, and the vein had to be tied.   The ether was discontinued and the rat taken from the bath.   Within 10 minutes it was walking about.   The surface tissues, now decolorizing, were still acidotic as shown by a *carrot red* color.

At 1.40 p.m. when the animal had completely decolorized a second injection of 1 cc. of phenol red was given.   By 2.30 it was deeply colored, *carnelian red*.   At 5.10 the injection was repeated.   And now the hue was indicative of a return toward the normal, being between *jasper red* and *coral red*.   During the afternoon the rat voided great quantities of intensely orange urine.   Next morning, as well as 5 days later, it was in excellent condition.   Healing of the neck incision was rapid.

Experiment II.—The rat weighed 124 gm. and received 1.85 cc. of the same phthalein solution used previously, soon after which it was etherized and placed in the oil bath.   In the course of 1 hour and 40 minutes 14.8 cc. of N/6.35 HCl was run into a jugular vein.   Toward the latter part of this period there was marked air hunger.   20 minutes after the injection,—and the ether,—had been terminated, with the animal already up and about, it was given 1.0 cc. more of the phthalein into the peritoneal cavity.   Within a minute it died, as if from an embolus,—though none could be found in the pulmonary artery.   A clot was present in the jugular below the tie.   The color changes during the injection of acid had followed the general course of those noted in Experiment I.

Experiment III.—The test was repeated but in this instance the rat of 128 gm. was anesthetized with 1.1 cc. of 20 per cent urethane injected into the subcutaneous tissue of the back of the neck.   15 minutes later it received into the peritoneal cavity 1.9 cc. of 4 per cent phenol red at pH 7.4.   When the injection of acid was begun 47 minutes had elapsed in all.   During the course of the next 2 hours and 24 minutes 13.5 cc. was run in.   The final hue was markedly acidotic, between *light ochraceous-salmon* and *light ochraceous-buff*.   The animal died about an hour later.   There was no respiratory distress at any time, in noteworthy contrast to the air hunger seen when such an experiment is done under ether.

Other similar observations were made which confirmed the findings described.   When N/6.35 HCl is injected (3) the connective tissue and tendons change from yellow-pink and pinky yellow respectively to a clear orange-yellow.   When the rats are stained with brom cresol purple instead of phenol red a change can be seen in the color of the cartilage, evidencing that it also has been rendered acidotic.

·The Vital Staining of Rabbits with Phenol Red.

White rabbits can be brilliantly stained with phenol red by the injection into an ear vein of 5 cc. of a 4 per cent solution for every kilo of body weight. Within half an hour the body surface is an intense red, but soon afterwards decolorization begins, so rapidly is the dye eliminated. The animal behaves normally from first to last, and can be stained again and again without obvious injury. Most of the phthalein—as much as 96 per cent—appears in the urine within 24 hours of the injection.

The chest and abdomen of the rabbits were shaved a day or so beforehand in order to lessen the possibilities of error due to inflammation from abrasions. They were placed upon an electrically heated pad and were often covered lightly with a cloth to ensure further against an abnormal loss of body heat. The same apparatus was used to warm the injection fluid as in the case of rats but the tube leading to the vein passed directly from the water jacket to the cannula only a few centimeters away.

The color of the shaved body of the vitally stained rabbit tends to be slightly more alkaline than that of rats similarly treated. It varies with the individual from a very exceptional *jasper red* (Ridgway)[1] to *eugenia red* more commonly. The color changes can be appraised for purposes of record in terms of pH by a method already briefly described (4). An Autenrieth wedge filled with water is placed over the shaved and oiled surface of the abdomen or chest, care being taken not to exert pressure, and the color as thus viewed is compared with that noted when one or another of a series of similar wedges, filled with buffer solutions of known pH, colored with phenol red, is placed over the shaved surface of a control rabbit. By viewing through a slit the color of the various portions of the wedge placed on the control it is possible to obtain a match in color intensity as well as color character. Differences of much less than 0.1 pH can easily be read in this way. The wedges should be warmed to 35–38°C. The hue of the vitally stained but otherwise normal animal, as expressed on the pH scale, varies between pH 7.4 (*jasper red*) and pH 7.6+ (a hue slightly purpler than *eugenia red*). It will be seen that these individual variations have about the same magnitude as those which are known to occur in the normal blood. But needless to say our

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[1] Ridgway's "Color standards and nomenclature" (Published by the Author, Washington, D. C., 1912) has been utilized in recording the colors, as in a previous paper of this series (Rous, P., *J. Exp. Med.*, 1926, xliv, 815). Wherever the hues provided by this book are mentioned in describing the findings italics will be employed.

use of pH figures to record the surface hue must not be taken to imply that the visible tissues have actually this pH, since there are many factors of complication. For one thing the surface is viewed by reflected light whereas readings of actual pH should be accomplished by transmitted light. The color of a fold of loose skin as seen by the latter method when it is pulled away from the body wall by gentle traction is of a different red from that observed on inspection of the same skin *in situ* by reflected light. It might be thought that the deeply stained blood would supply an important part of the surface color; but this is not the case. When all blood is washed out of the animal the surface is still an intense red (5); and whole thickness skin grafts stain heavily with the phthalein even during the period when they are completely avascular (6). One can safely conclude that the hue of the intact body surface in the living animal is referable to a distribution of the dye to the tissues; and it is upon this well justified assumption that our experiments are based. The precise whereabouts of the extravascular phthalein that renders the animal ruddy is another matter. So little of it is in the epidermis that this may be neglected; but much is in the tissue fluid,—as can be sufficiently shown by the change in color witnessed when the latter is forced out, as by compressing a skin fold between glass slides. A great deal is fixed upon the interstitial elements of the connective tissue (7). Whether the cells of this tissue contain any is doubtful.

To follow the changes in hue of the connective tissue, as distinct from those in the composite color of the body surface we have run a fine thread through the oiled skin here and there in regions where it was loose, as just above the groins; and at various periods of the experiment by pulling upon the thread have raised a fold and compressed it briefly between slides. The color of the cartilage could be followed in the ears when the circulation in them was good; but not infrequently it was so poor that the initial staining greatly lagged behind that of the body generally, as, too, did the changes consequent upon the administration of acid. In the rat the normal cartilage stains orange-yellow with phenol red, whereas in the rabbit it becomes pale pink. The more considerable alkalinity thus evidenced in the case of the latter animal is also encountered in the connective tissue which, in a compressed skin fold, appears a frank pink with only the faintest suggestion of yellow, not yellow-pink as in the rat.

By the technic described we were able to follow closely the extravascular changes in surface hue indicative of *relative* changes in pH; and, more roughly, the changes in color undergone by connective tissue and cartilage *in situ*. It remained to determine the reaction of the blood. This was accomplished by Hawkins' method (8).

The blood specimens were taken from the marginal ear vein directly into pipettes graduated to 1/100 cc., which had been ground to fit Luer needles. The latter were short, of 23 gauge, and they had been bent at a reentrant angle for con-

venience in piercing the vein in the direction of the ear tip. At the moment when this was to be done the operator made pressure upon the vein distal to the point of entry of the needle, releasing it at once thereafter so that the flow might be unimpeded. Since the vessels were distended by stroking or heat the specimens (0.25 cc.) must be thought of as consisting for the most part of arterial blood. They were procured in duplicate under oil and diluted in the usual way with salt solution. When the animal was heavily stained only enough phenol red had to be added to this solution to permit of the preliminary adjustment to pH 7.4. More of the phthalein proved necessary, of course, when the rabbit happened to be decolorizing. The protective layer of paraffin oil was replaced with melted paraffin (of low melting point) as soon as the blood and salt solution had been mixed. Readings were made in a colorimeter block at 38°C. The correspondence between the two tubes of any given specimen was, in our experience, absolute; and the whole procedure proved easy to carry out. The pipettes and needles required most careful cleansing else clotting was encountered. The same vein could be pierced again and again. This was essential to avoid using the ear into which phthalein had been injected.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

The Outlying Acidosis Attending Ether or Urethane Anesthesia.

In initial tests the rabbits were anesthetized with ether or urethane and they lay on the back throughout the injection period. Even under the best of conditions and in the absence of anesthesia the mere weight of the body proved sufficient to give rise to an acidosis of the surface tissues pressed upon, as was shown by their change in color from red to orange; and when the limbs were stretched out under tension the circulation on the surface of the abdomen was frequently interfered with and some acidosis developed there as well. But even in the absence of these sources of disturbance there was frequently a local acidosis to be observed in the anesthetized rabbits. On injection with phenol red after they had been unconscious for some time they colored up much less promptly than did normal animals; and large irregular regions over the body and in the ears remained untinted after the red staining had become pronounced everywhere else. When phthalein did at length penetrate into these regions their color had an orange cast. Evidently a local acidosis had developed in them, secondarily to an interference with the blood supply. As

is well known a blood acidosis develops during ether anesthesia. But independently of any such change, there was an extravascular acidosis, an "outlying acidosis" resembling that we have described in other connections (9).

Experiment IV.—Effects of urethane. 142 cc. of N/6.35 HCl was run into the jugular vein of a male white rabbit (No. 1) weighing 1625 gm., which had been given 3 cc. of 50 per cent urethane into the peritoneal cavity 2 hours beforehand. The injection took 1 hour and 40 minutes. When 50 cc. had been given 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 5.6 was slowly introduced into an ear vein, without intermitting the flow of acid to the jugular. The animal stained only very gradually and irregularly *jasper red*, with patches coloring up later of *coral red* here and there. As time passed and the HCl injection proceeded the color altered more and more toward orange, and had become *rufous* in some places, *apricot orange* in others, by the time all the acid was in. The determinations on the blood showed a fall in pH from 7.4 prior to the giving of the acid to 7.0 immediately afterwards. Later the surface color became still yellower, between *zinc orange* and *ochraceous-salmon*, instead of altering toward red, and the animal died 1 hour and 40 minutes after the injection had been finished. From first to last there was no air hunger despite the acidosis. The surface hue at the end corresponded with pH 6.8.

In this instance not only was the staining irregular, with relatively acidotic areas here and there, but the complete absence of a compensatory increase in the breathing attested to a depressant effect of the urethane. To the absence of this respiratory compensation death was attributable, the animal becoming more and more acidotic after the injection was stopped, instead of tending to recover as happens after ether or in the absence of any general anesthesia. Cushny and Lieb have shown that the reaction of the respiratory center is altered in rabbits deeply under urethane (10).

Experiment V.—Effects of ether. The rabbit (No. 3) weighed 1600 gm. The *first blood specimens*, taken just prior to a light etherization, were at pH 7.4+. Now the jugular vein was cannulated, and 35 minutes after the blood had been taken the acid injection was begun. When 71 cc. of N/6.35 HCl had been run in the cannula was flushed with salt solution by means of a three-way stop-cock connecting with it, and 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 7.4 was gradually injected. The animal colored only very gradually, *jasper red* here, *coral red* there, with a streak of orange-yellow along the midline from ensiform to symphysis. As more and more acid was injected the respirations became stormy and the general color changed to *apricot orange* variegated with

rufous. After 160 cc., given in the course of 2 hours, the jugular was tied and the ether, which for some time had been almost left off, was wholly discontinued. The *second blood specimens* were now taken, reading midway between pH 6.8 and 6.9. The *surface hue* on the other hand corresponded with 7.0− to 7.0+. The animal soon got to its feet and shortly thereafter the staining lost its patchy character. During the rest of the day it appeared in good condition save for the exaggerated respirations. Several blood specimens were taken. These showed only a slight return toward a normal reaction whereas there was a more marked extravascular return as shown by the surface color. The changes find record in the chart (Chart 1).

The animal was given food and left for the night. Next morning it was reported to be decolorized and in good condition, having eaten well. Unfortunately it was placed in a cage with other animals to be brought to the laboratory, and, as it moved about amongst them, it suddenly collapsed and died, making violent respiratory efforts. Apparently its slight exertions had been sufficient to overturn the *status quo.* At autopsy no anatomical cause for death was found.

In this experiment (Chart 1), which was duplicated by another, the injection of an enormous amount of the acid solution was withstood by the animal. There was the stormy respiration usual in acidosis, a feature which had been lacking with urethane, and after the injection the animal tended to recover as had not been the case when the latter was used. The staining with phenol red, done at a time when considerable acid had been run in, disclosed a poor circulatory condition of the tissues, as shown by a tardy distribution of the dye and the presence of relatively acidotic areas over chest and abdomen. The rapid change to an even staining after the animal had come out of the anesthetic was a significant feature as was also the return of the surface color toward the normal during a period when the blood reaction remained almost unchanged. As the later work showed, this return was referable to the disappearance, as the circulation became better, of a generalized "outlying acidosis" which had been present in addition to the extravascular acidosis induced by the HCl. In none of the later experiments, in which a local anesthesia took the place of a general one, was any pronounced color patching noted like that with ether or urethane; in none did the hue become as orange,—despite the fact that the injection of acid was pushed to the extreme; and in none did the extravascular reaction return more rapidly to the normal than that of the blood.
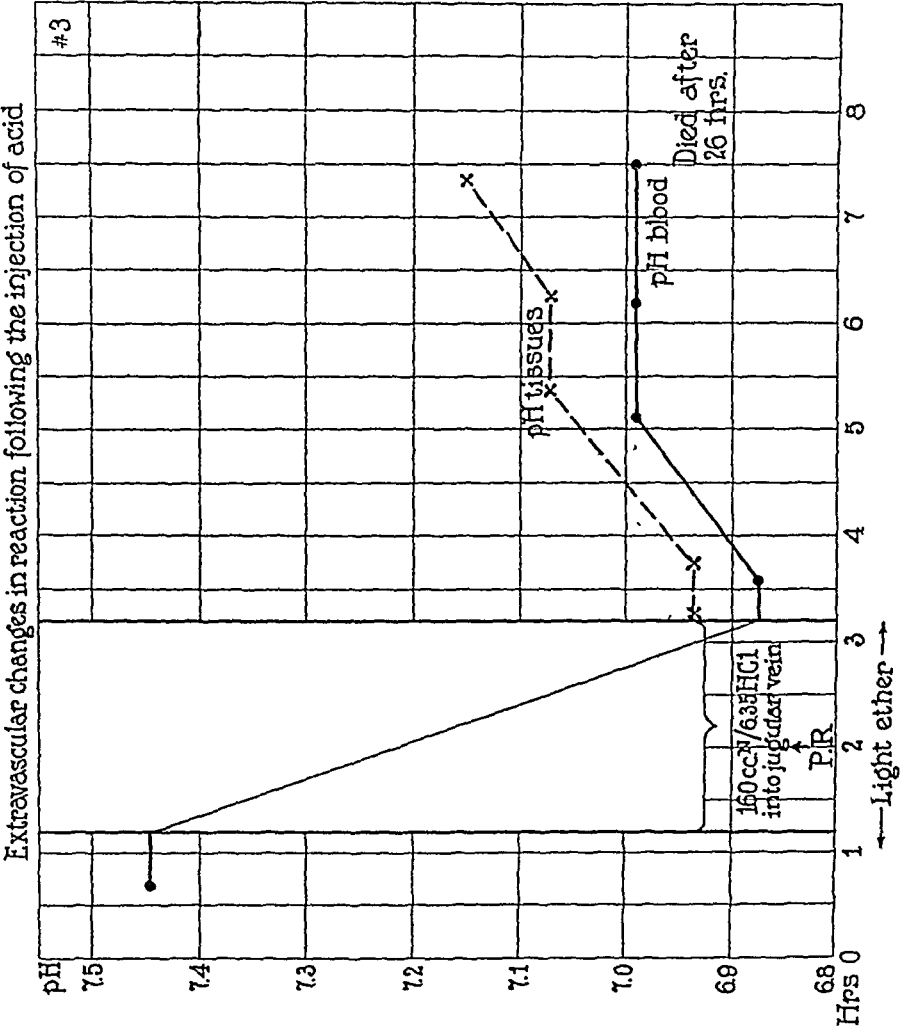
CHART 1.

For the purpose of the foregoing observations the animals were stained with phenol red only after the injection of acid had proceeded for some time; since otherwise the staining did not last long enough to cover the entire later period of study. The plan was now adopted of giving smaller amounts of the indicator with repetitions of the dose at more frequent intervals. Tests directed to the point had shown that the intravenous injection of 10 cc. of 4 per cent phenol red, brought to pH 7.4, exerted not the slightest effect on the reaction of the blood. To expose the jugular vein novocaine was employed, without precautions for sterility. After the injection was finished the neck incision was sutured and the animal placed by itself in a metabolism cage where it could be carefully watched. Abstracts of two protocols will be given.

Experiment VI.—(See Chart 2.) Male white rabbit (No. 6) of 1690 gm. 9.30 a.m.—*First blood specimens taken.* 9.37—First injection of phenol red. 10.05—Rabbit coloring rapidly and evenly. 10.22.—*Surface hue is old rose;* by wedge method pH 7.5. 10.30—*Second blood specimens taken.* 11.29—*Injection of N/6.35 HCl begun* at rate of 5 cc. every 3 minutes. 12.06 p.m.—65 cc. in. Animal completely decolorized. 12.35—102 cc. in. Pause for injection of phenol red, 10 cc., by way of the jugular cannula, between 12.36 and 12.39 p.m. Injection of acid now renewed at a slower rate. 12.56—115 cc. of acid has been injected. The animal is everywhere *jasper red.* 2.29—The general color is *coral red* but with carroty areas here and there. *Injection terminated and animal removed from pad.* It has received 170 cc. of acid in all. 2.50—*Third blood specimens taken.* Animal O.K. 3.00—*Surface hue* between *coral red* and *carnelian red;* by wedge method pH 7.2. 4.33—*Fourth blood specimens taken.* 4.39-40—Injection of phenol red, 5 cc., into an ear vein. 5.13—*Surface hue* by wedge method pH 7.3−. 5.18—*Fifth blood specimens taken.* 5.41—*Surface hue* by wedge method just about pH 7.3 (artificial light).

During the latter part of the period of injection the respiratory amplitude was greatly increased. The animal remained quiet throughout, however. Afterwards it voided large quantities of urine colored orange-red with phthalein. During the night it escaped from the metabolism cage, and some urine may have been lost. Next morning the respirations were still as exaggerated as on the previous evening, and the animal was weak and apathetic.

Second Day, 9.55 a.m.—*Sixth blood specimen taken.* 10.08—Injection of phenol red, 5 cc. into an ear vein. 10.20—The rabbit has colored but poorly, yet there is no evident outlying acidosis. 10.39—The coloration is diffuse; *surface hue* by wedge method, pH 7.3+. 11.18—*Seventh blood specimen taken.* 11.33—*Surface hue* by wedge method, pH 7.3. Respirations still exaggerated. 12.07—There is

a sudden commotion in the cage, and the rabbit previously quiet, is found in opisthotonos. It died at 12.10.

The autopsy showed atelectasis of the upper lobes of both lungs, and in the right psoas muscle a contracted blood clot apparently about 24 hours old with a volume of about 10 cc.

Extravascular changes in reaction following the injection of acid
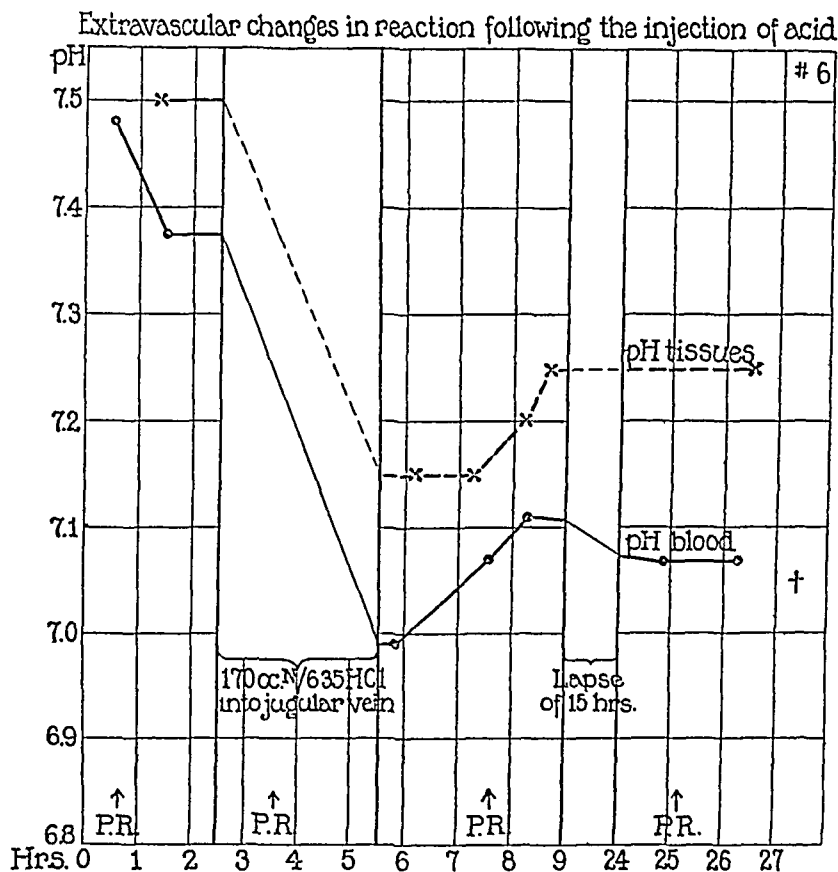
CHART 2.

The bladder had been emptied by catheterization at the beginning of the experiment. From then until death 184 cc. of urine was secreted, an amount somewhat larger than the total of acid solution injected. The rabbit had free access to water during the night and to food but appeared not to have partaken of either. The amount of circulating hemoglobin fell from 78 per cent to 58 per cent (Newcomer), and the red cells from 6,590,000 to 3,490,000 per c.mm. in the first 24 hours. A part of the blood change is attributable to the hemorrhage into the muscle.

In this experiment the maximum quantity of acid was given that could be borne. The reaction of the blood was altered from pH 7.4 to pH 7.0, and during the next 24 hours it scarcely changed (Chart 2). The surface color, recorded in terms of pH, gave readings that were regularly further on the alkaline side than those of the blood; but, as has already been emphasized, these readings cannot under the conditions be taken as expressive of the actual pH prevailing. They have merely a relative value as indicative of the extravascular condition. The change in this would appear to have been not quite so great as in the blood, the apparent pH falling from about pH 7.5 to pH 7.2. The later, slight recovery in the blood was duplicated, however, by an extravascular recovery of equal magnitude.

The death of the animal cannot be attributed directly to the acidosis because of the lesions found at autopsy. The question comes up, indeed, as to whether the persistence of the acidosis may not have been due to these latter. That it was not, the following experiment shows.

Experiment VII.—Male rabbit (No. 7) of 1650 gm. Jugular bared under local anesthesia with novocaine. 9.42—*First blood specimens taken.* 9.50½-52— Injection into an ear vein of 4½ cc. phenol red. 10.22—*Surface hue* between *old rose* and *eugenia red;* by wedge method, pH 7.5. 10.53—*Second blood specimens taken.* 11.02—*Surface hue* by wedge method pH 7.5. 11.46—*Injection of* N/6.35 *HCl begun.* 12.36 p.m.—59 cc. has been injected. 1.13—Injection of 5 cc. phenol red by way of the cannula in the jugular. The animal colored evenly and quickly, *jasper red.* 1.46 p.m.—132 cc. of acid in. *Surface hue* between *light jasper red* and *coral red.* 2.02 p.m.—142 cc. of acid in. Rabbit is restless; breathing greatly exaggerated. 2.19—Animal nearly decolorized. 2.24 p.m.— 149 cc. in. 2.24-26½—10 cc. of phenol red injected into jugular. A rapid and deep staining developed. 3.01 p.m.—158½ cc. of acid has been run in; *injection terminated* and incision sewed up. 3.10—*Surface hue* between *coral red* and *jasper red.* 3.11—Rabbit removed from board. It is alert but stands weakly, and has a notable air hunger. 3.26—In excellent condition, though still weak and with air hunger. *Surface hue* now slightly darker than *carrot red.* 3.43— *Surface hue* by wedge method between pH 7.1 and pH 7.2. 3.52—*Third blood specimens taken.* 4.00—*Surface hue* again between pH 7.1 and pH 7.2. 4.51— *Surface hue* just above pH 7.1. 4.57—*Fourth blood specimens taken.* 5.03— *Surface hue* between pH 7.1 and 7.2. 5.20—Decolorizing. Left overnight, with water but no food. Eager to eat cabbage shown it.

Second Day. 9.35 a.m.—Animal in excellent condition except for weakness of fore leg on side of jugular injection. Breathing still exaggerated. 9.44—*Fifth*
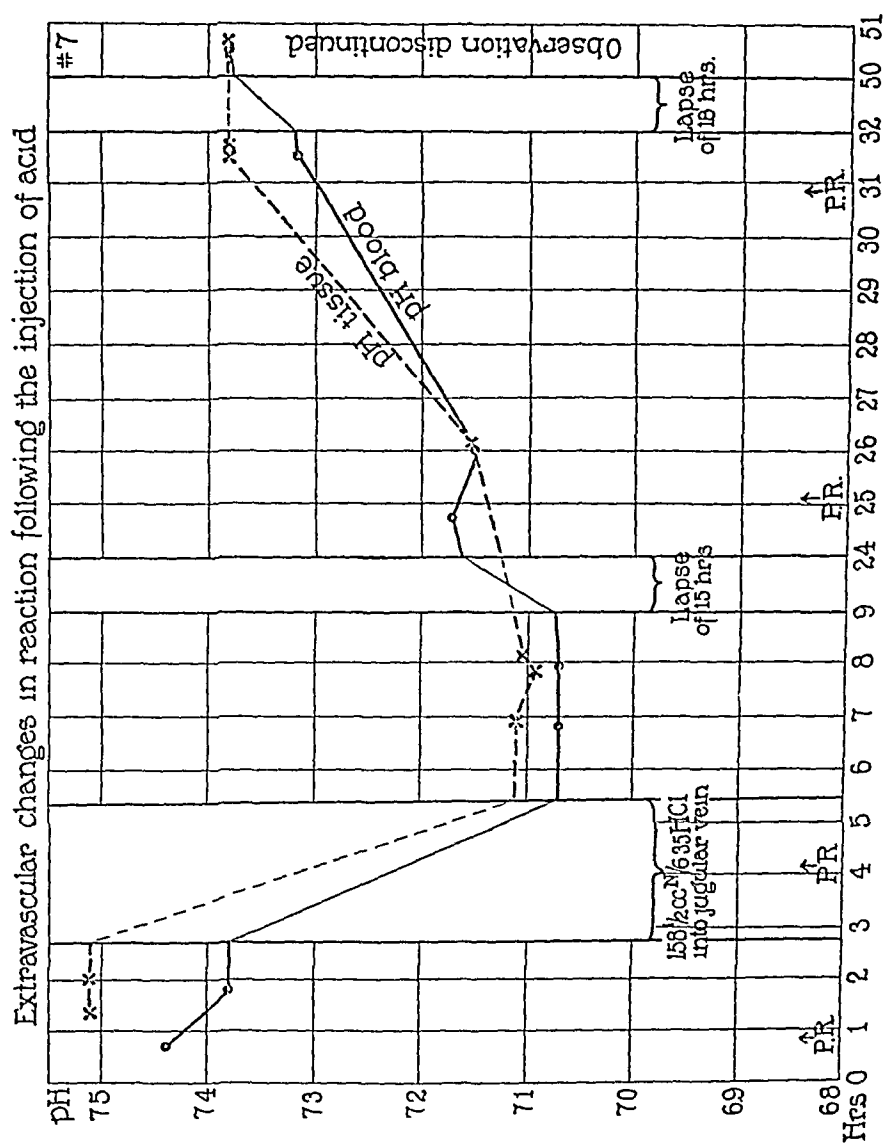
CHART 3.

blood specimens taken. 10.04-06—7 cc. of phenol red injected into an ear vein. The staining that followed was rapid, even, and deep; *surface hue* by wedge method pH 7.3. 10.41—*Surface hue,* by wedge, just below pH 7.3. 11.00—*Sixth blood specimens taken.* 11.04—*Surface hue* by wedge, pH 7.2. 11.09—Color is between *light jasper red* and *light coral red,* slightly darker than either. 11.32—Given cabbage which it eagerly attacks. 2.15—Has eaten largely; condition excellent though right fore leg is still dragged. 3.53—Injected with 7 cc. of phenol red. 4.14—General color *jasper red.* 4.32—*Surface hue* by wedge, pH 7.4. 4.35—*Seventh blood specimens taken.* Still some air hunger on exertion. 4.42—*Surface hue* pH 7.4. 6.13—Left overnight with food. General condition as before.

Third Day. 10.24 a.m.—Has again eaten largely. Injected with 7 cc. phenol red. 10.35—Given more cabbage; eating. 11.07—*Surface hue* by wedge pH 7.4, and again pH 7.4 at 11.24. 11.28—*Eighth blood specimens taken. Surface hue* by wedge pH 7.4. Decolorizing; color is *old rose.* 12.27—Not yet fully decolorized; observations terminated.

Fourth Day. Weight 1500 gm.

Fifth Day. The tissues of the neck are indurated about the old wound. Animal killed with chloroform. The general condition of the organs is excellent save in in the case of the kidneys, the renal cortex appearing somewhat swollen and paler than normal (parenchymatous degeneration?).

The bladder had been emptied by catheterization prior to the injection of acid, and during the 19 hours thereafter 223 cc. of urine heavily colored with phthalein was voided. During this period no food had been given.

The hemoglobin dropped from 75 per cent prior to the experiment to 58 per cent on the next morning, and the number of red cells from 5,460,000 to 4,430,000. In the afternoon the hemoglobin percentage was 65 and the number of red cells 4,750,000. On the 2nd day the figures were the same. For the determinations and those of Experiment VI we are indebted to Dr. Charles A. Doan.

In this experiment (Chart 3) the alteration in the surface color, as recorded in terms of pH, was slightly greater than that found in the blood, whereas in the preceding one it had been slightly less. The correspondence between the later extravascular changes, indicated by the surface hue, and the intravascular ones was exceedingly close. The observer following the alterations in the blood did not know of those in the surface hue. The variations in pH were almost identically the same in both, and the same very gradual recovery took place until food (cabbage) was given, when the acidosis rapidly lessened, almost disappearing within a few hours. How long recovery might have taken had no materials to combat the acidosis been supplied by mouth is problematic.

The amount of urine voided by the animal during the first 19 hours after the acid injection (223 cc.) considerably exceeded the fluid bulk introduced in this way (158½ cc.).

The diminution in the amount of blood in circulation attested by the lessening in hemoglobin and in the number of circulating red cells is to be attributed in no small part to the repeated bleedings incident to the observations on the blood reaction. The remaining loss was doubtless due to an action of the hydrochloric acid solution on such red cells as came into direct contact with it during the injection period.

Significance of the Extravascular Changes.

The alterations in the surface color that took place during the foregoing experiments might be attributed wholly to changes in the reaction of the stained extravascular fluid did not incidental observations show that the hue of the tissues themselves was altered. We have already described the method whereby it is possible to determine the hue of connective tissue *in situ*. Oiled folds of skin of the vitally stained rabbit when viewed between slides by transmitted light are ordinarily pink even when the fluid has been driven out of them by pressure upon the slides. After the injection of acid they appeared orange-pink until pressure was made when they were seen to be of a clear orange-yellow, far more acid, judging from the color, than the body surface as viewed by reflected light. The conjunctiva likewise became orange, and the sclera palely so. The cartilage in the least vascular portions of the ears of vitally stained rabbits is pink ordinarily. But after the acid injection it was a clear orange-yellow. In proportion as the general acidosis was recovered from, so too did all of the tissues mentioned alter from orange to pink. On the inside of the ear where the cartilage is covered with merely a film of adherent skin its color can be readily followed by reflected light; and the observation was made that at times when the alteration in the hue of the body surface generally had progressed no further than *coral red* the cartilage had become a clear orange. The finding would suggest that despite the relative alkalinity of normal rabbit cartilage as compared with that of the rat or mouse, an alkalinity finding expression in a pink staining with phenol red,

this tissue is still not so alkaline as the connective tissue, which in turn is less alkaline than the blood (11). The pH of normal rabbit blood is almost identical with that of the rat (12).

DISCUSSION.

The findings show that when changes in the reaction of rabbit blood are induced with hydrochloric acid extravascular changes of approximately the same magnitude occur. No dissections were made to inspect the interior organs; but from previous experiments on rats it seems certain that conditions of blood acidosis or alkalosis leading to a change in the color of connective tissue and cartilage will also lead to one in the color of the tendons, whereas the hue of liver, pancreas, and lymph nodes will be unaffected even when the blood change is so great as to lead to death (13). There is every reason to suppose that the same holds true in the case of rabbits, and that by following the visible alterations in the connective tissue and cartilage one can obtain an index to much of what would be disclosed by dissection under oil.

The failure of the extravascular reaction to alter more than does that of the blood would at first sight seem surprising. For, with a lessened gradient between tissues and blood, one might suppose that there would be some heaping up of the acid products of metabolic activity. Perhaps such a heaping up and not a passage of H ions from the blood is actually one of the causes of the extravascular acidosis. Whenever the circulation is interfered with, as during ether and urethane anesthesia, a heaping up of acid metabolites certainly does occur, with result in an "outlying acidosis" independent of that due to the injected acid. One is reminded in this connection of Yandell Henderson's statement that most patients and animals under general anesthesia for more than an hour are in the first stage of shock (14). Our experiments prove that under the conditions of such anesthesia an abnormal local reaction develops within some of the tissues which may well prove detrimental to the organism as a whole.

The demonstration that a widespread extravascular acidosis occurs when there is a marked blood acidosis, and that it is tolerated for

long periods has a bearing upon the problem of shock. For many years now the relationship between the functional state of the small blood vessels and the reaction of their milieu has been debated; and many have supposed that a vascular dilatation is caused by acidosis and that such a dilatation may be responsible for shock through the changes in the distribution of blood that it entails. The British Commission for the investigation of surgical shock could not produce it by injecting acid into the circulation (15); but they were unable to bring proof that in altering the condition of the blood they also altered the reaction outside of the lumen of the vessels. That extra-vascular changes go hand in hand with those taking place in the blood has been demonstrated in the course of the present work. Yet not the least evidence of shock was to be seen in our animals though the blood reaction was diminished to the limit compatible with life and the extravascular reaction of large regions was proportionately affected. Though the connective tissue was markedly acidotic, as shown by its color during the periods of staining, not the least vascular dilatation could be observed in it through the shaved and oiled skin. In the intervals of decolorization between the injections of phenol red there was good opportunity to make observations on the latter point.

The quantities of fluid voided by our animals during the many hours that the tissue acidosis endured regularly exceeded the very large amounts introduced with the acid. These animals had access to water but were given no food, and they certainly did not drink largely. There was not the least sign of a fluid retention in the acidotic tissues. In a previous paper (16) one of us has demonstrated that local acidosis and edema sometimes occur together but without any relationship of cause and effect. The present findings afford another example of the phenomenon.

SUMMARY.

The changes in blood reaction caused by the injection into a vein of a weak solution of hydrochloric acid are accompanied by extra-vascular changes of similar magnitude within the subcutaneous tissue. Under the conditions of prolonged general anesthesia with

ether or urethane the circulation to this tissue is so interfered with that an "outlying acidosis" may develop in addition to the acidosis immediately consequent on the blood state. Even under the best of circumstances the extravascular acidosis induced with hydrochloric acid affects not merely the tissue fluid but the reaction of the tissue itself.

Rabbits in which a widespread extravascular acidosis has been produced, together with a blood acidosis as severe as is compatible with life, remain in good condition during the relatively long period over which this state of affairs persists. There is at no time any sign of capillary dilatation, though the vessels are bathed in relatively acid fluid, and none of shock. No edema develops in the acidotic tissues, and the animals void large amounts of urine. The tissue acidosis lessens *pari passu* with that of the blood.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

IX. On the Tissue Reaction as Influenced by Inhalations of CO_2 and by Overbreathing.

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In preceding papers from this laboratory the influence upon the tissue reaction of injections into the circulation of weak solutions of acid and alkali has been recorded, and the relation existing between the blood changes and those in the tissues during the course of an acidosis induced in this way has been described (1). We have now sought to determine the effect upon the tissues of breathing high concentrations of carbon dioxide, and of the overventilation that results from forced respiration.

The Inhalation Apparatus.

White rabbits were used. The general technic of vital staining with phenol red and of appraising the reaction of the blood and the changes in the color of the body surface have already been detailed (2). The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

A special apparatus was necessary, for the purpose of the inhalations of carbon dioxide. It is portrayed in Text-fig. 1.

The gas from the storage cylinder escaped into one or the other of two 20 litre bottles graduated in half litres and about half full of water weakly acidulated with hydrochloric acid. The pressure under which the gas passed into the one bottle was sufficient to force water over into the other through the large tube connecting the two, and the only way of escape for the carbon dioxide already in this other was through a tube which led to the inhalation mask fixed upon the animal's muzzle. By means of a special snap clamp (X) acting, now on the tubes A, and again on B, the direction of flow from the storage cylinder could be alternated to

the bottles at will without interruption of it.   An observer with a stop-watch controlled the speed at which gas passed to the inhalation mask.   One litre per minute was the ordinary rate.

The mask was made out of a short broad bottle from which the bottom had been cut.   Across the opening thus provided a diaphragm of rubber was stretched with a hole in the center for the rabbit's muzzle.   There was a continuous current of gas through the bottle, and the space in it was small, to prevent rebreathing. The tube through which the gas escaped was long in order that there might be no sucking back of air into the mask on inspiration.   To ensure a snug fit of the rubber diaphragm all hair was removed from the animal's muzzle with a sodium sulfide solution.   The mask was held in place by a strip of adhesive tape passing
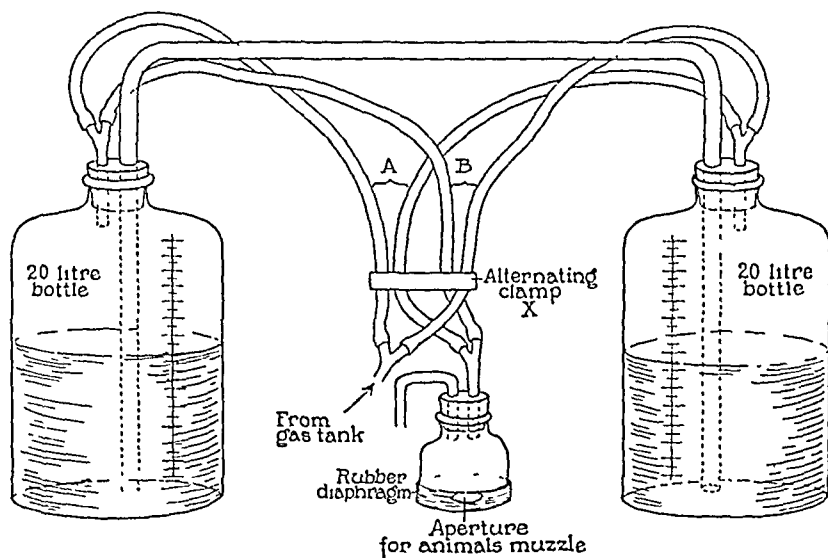
TEXT-FIG. 1.

around the back of the head, and there was never any leakage between it and the skin.   Care was taken not to interfere with the circulation to the ears.

Five experiments were performed with a mixture containing 21.08 per cent of CO_2, and approximately 22.09 per cent O, and 56.83 per cent N.   A single set of observations were made with 37 per cent CO_2, 22 per cent O and 41 per cent N.   For the analyses of the gas mixtures, as supplied in pressure cylinders, we are indebted to the kindness of Dr. C. A. L. Binger.

In four of the five experiments just referred to, urethane was employed as anes-thetic.   The blood specimens for the pH determinations were procured from a

femoral vein. In the case of the remaining animal, which received no general anesthetic the small incision necessary to bare the vein was made after infiltration of the tissues with novocaine. To aspirate blood Luer needles bent at a reentrant angle were used, as in the previous work. It was essential that the circulation of the leg furnishing the blood should not be interfered with, so no thong or other tie was placed about it. The vein could be punctured again and again in the groin without any important bleeding or thrombosis, if the needle was thrust slantingly through its sheath and pressure applied for a few moments after the aspiration.

In several of the experiments a minimum dose of urethane was employed and a far better respiratory response to the acidosis was obtained than in the animals more heavily anesthetized by its means and given hydrochloric acid (3).

The Acidosis on Breathing 21 Per Cent of CO_2.

The protocol of *Experiment 1* need not be given since the facts are better illustrated by the later work.

Experiment 2.—Male rabbit No. 2, of 1650 gm., given 3.3 cc. of 50 per cent urethane subcutaneously into the neck at 9.20 a.m., and 1 cc. more at 10.35, into the peritoneal cavity. 11.00—10 cc. of 4 per cent phenol red isotonic with 0.9 per cent NaCl and at pH 7.4 was injected into an ear vein. 11.50—Rabbit for some time on the warm pad. *Surface hue* slightly less purple than *eugenia red* (Ridgway); by wedge method between pH 7.5 and 7.6. Skin folds compressed between slides are pink by transmitted light with faintest yellow admixture; ear cartilage pale pink. 11.54—*First blood specimens taken.* 12.00—*Surface hue* between pH 7.5 and 7.6.

12.01—*Mask adjusted and gas run in.* The respirations at once mounted from 40 to 88 per minute, much exaggerated; but almost as rapidly they lessened in frequency again.

12.05—*The surface hue* generally has become yellower, between *jasper red* and *light jasper red*. No indication of spotting. 12.07—Respirations 56 per minute, regular. 12.10—*Surface hue* yellower than *jasper red*. Skin folds orange-yellow. 12.12—*Surface hue coral red*. 12.41—*Surface hue light coral red*. Skin folds pronouncedly orange-yellow; ear cartilage light yellow. 12.48—*Surface hue* by wedge method, pH 7.2. 12.51—*Second blood specimens taken.* 12.55—*Surface hue* by wedge, pH 7.2. 12.56—*Surface hue* still *light coral red*. During this long inhalation period the respirations have been much exaggerated but never stormy. Their rate has lessened from 56 to 48 per minute.

12.57—*Mask removed.* The respirations at once became much less ample, 52 per minute. Practically at once, too, the *surface hue* became pinker, the change being very definite by 12.59.

1.01—Animal stirred, as it had not during the inhalations. 1.03—Skin fold much less yellow. 1.08—*Surface hue* now approaches *old rose*. 1.12—*Surface hue* by wedge, pH 7.4. 1.14—Ear cartilage more pink than yellow now, while skin fold is as pink as before the inhalations. 1.19—*Surface hue* between *jasper pink* and *old rose*, pH 7.4 by wedge. 1.26—*Third blood specimens taken*. 1.30—*Surface hue* by wedge pH 7.4 —; between *light jasper red* and *jasper pink*. Observations discontinued. The respirations varied between 52 and 40 after the mask was off; they were quiet.

Experiment 3.—Performed on afternoon of same day as Experiment 2 and upon the same animal, which was still unconscious owing to the urethane. 2.52—Given 8 cc. of phenol red into the same ear vein as before. 3.17—*Surface hue* slightly purpler and deeper than *jasper red*, by wedge method pH 7.5 +. 3.19—*First blood specimens taken*. Ear cartilage is light pink; skin folds, compressed, are pink, with faintest yellow tinge. 3.23—*Surface hue* by wedge, pH 7.5 +.

3.28—*Mask adjusted and inhalations begun.* The respirations, which had averaged 48 per minute, quickened greatly at once but soon slowed again, to 62, much exaggerated.
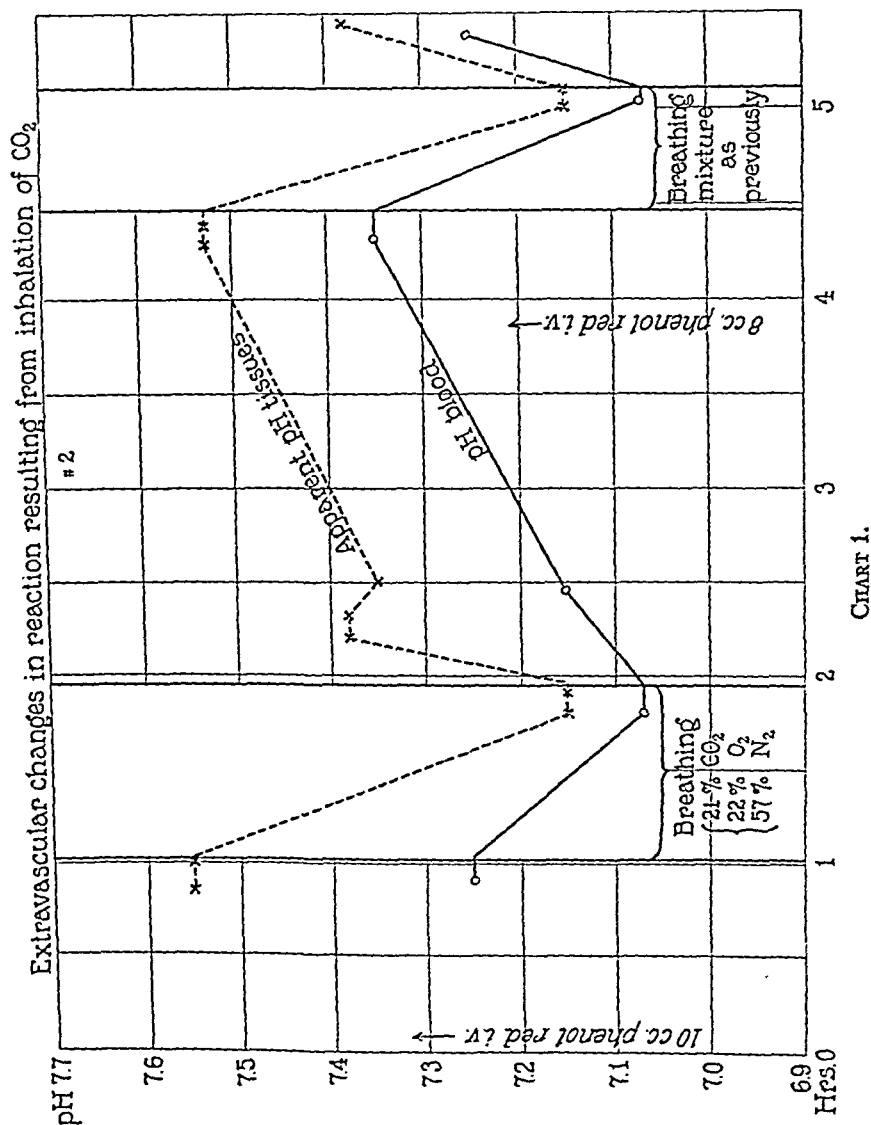
3.32—*Surface hue* yellower. 3.33—Skin folds much yellower. Some surface patching of color. 3.35—The general color is *jasper red*, with some *coral red* patching over chest and abdomen. The patches are irregular, only a few centimeters across. They have the general character of those seen in outlying acidosis. 3.43—The *coral red* patches have become confluent, everywhere driving out the rosy hue. A skin fold is bright orange-yellow but the color of the ear cartilage has not changed. 3.53—*Surface hue* pH 7.2 + by wedge method. There is a definite yellowing of the ear cartilage. The circulation in the ear is excellent. 4.00—*Surface hue* still that of pH 7.2 +. 4.02—*Second blood specimens taken.* 4.07—*Surface hue* between *coral red* and *light coral red;* by wedge method pH 7.2 +. Ear cartilage yellow. During the inhalations the respirations gradually slowed from 62 to 44 per minute, markedly exaggerated always.

4.08—*Mask removed.* Immediately the breathing became stormy and quick for a few moments, but then quieted and slowed to a rate of 48 per minute and gradually, by 4.30, to 32 per minute. The color of the animal at once began to change back to the normal, being definitely more ruddy by 4.11. The change was generalized.

4.13—*Surface hue* between *jasper red* and *light jasper red;* skin fold pink, with but a slight admixture of yellow; ear cartilage still yellowish. 4.19—*Surface hue* between *light jasper red* and *old rose*. 4.25—*Third blood specimens taken.* 4.28—*Surface hue* by wedge, pH 7.4. 4.30—Animal stirring slightly.

Observations discontinued. 50 cc. of warm water given by stomach tube. Next morning the rabbit was in excellent condition, eating.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly, on test with the potentiometer, from the calculated pH values. Corrections have been made for the differences in charting the results, whereas in the protocols the figures originally read off are given.

Extravascular changes in reaction resulting from inhalation of CO_2

CHART 1.

Breathing mixture as previously

8 cc. phenol red i.v. →

#2

Apparent pH tissues

pH blood

Breathing { 21 % CO_2 / 22 % O_2 / 57 % N_2

← 10 cc. phenol red i.v.

As the protocols and the chart show (Chart 1) there was in these experiments not only a great alteration in the reaction of the blood but one in the extravascular reaction as well, when the animal inhaled a gas mixture containing 21 per cent CO_2 and the normal quantity of oxygen. The changes in the surface color indicative of an increasing acidity began practically at once, and, progressing rapidly, reached a maximum some little time before the inhalations were discontinued. When this had been done an immediate return toward the normal took place, at first rapid, then more gradual. In Experiment 3 there was, as the general acidosis developed, a patching which resembled that of outlying acidosis.

Experiment 4.—Male rabbit No. 4 of 1700 gm. given 4.5 cc. of 50 per cent urethane into the peritoneal cavity at 9.20 a.m. and 10 cc. of 4 per cent phenol red into an ear vein at 10.04. 10.25—Staining is even and deep; animal on warm pad, deeply anesthetized. 10.48—*Surface hue* not quite so purple as *eugenia red;* by wedge method at pH 7.5. 10.54—*First blood specimens taken.* 10.57—*Surface hue* still at pH 7.5. A skin fold is pink with slightest tinge of yellow; ear cartilage pink.

11.08—*Inhalations begun.* Immediately the respirations increased in rate and amplitude.

11.12—*Surface hue* is yellower. 11.14—*Surface hue* now *jasper red* save for a longitudinal streak about 4 cm. wide extending from ensiform nearly to symphysis, which is still purply red. 11.15—Skin fold more yellow. Respirations still markedly exaggerated but of nearly the same rate as before the inhalations were begun, 48 as compared with a previous 44 to 46 per minute. The rate altered practically not at all from now until the mask was removed. 11.23—Dubious yellowing of ear cartilage. Good circulation in the ear. 11.29—*Surface hue* between *jasper red* and *coral red.* 11.33—Skin folds are much more yellow; and so too is the ear cartilage. 11.40—*Surface hue* generally is between *coral red* and *light coral red.* The median streak of differing color has disappeared. 11.48—Circulation to ear is excellent yet cartilage is orange-yellow, as also are the skin folds. 11.52—*Surface hue* by wedge method pH 7.2+. *Second blood specimens taken.* 11.59—*Surface hue* at pH 7.2; yellower than *coral red.* Color of skin folds and cartilage orange-yellow.

12.00—*Mask removed.* Respirations temporarily increased in amplitude and frequency, being 60 to the minute at 12.01 but soon becoming quiet and slowing to 40. Practically no change took place in the *surface hue* for a long time, however. At 12.37 it was still between *coral red* and *light coral red;* and at 12.40, by wedge method, at pH 7.2.

12.46—*Third blood specimens taken.* 12.49—*Surface hue* by wedge pH 7.2. 2.00 p.m.—Rabbit still deeply unconscious, an even *light jasper red.* Skin surface
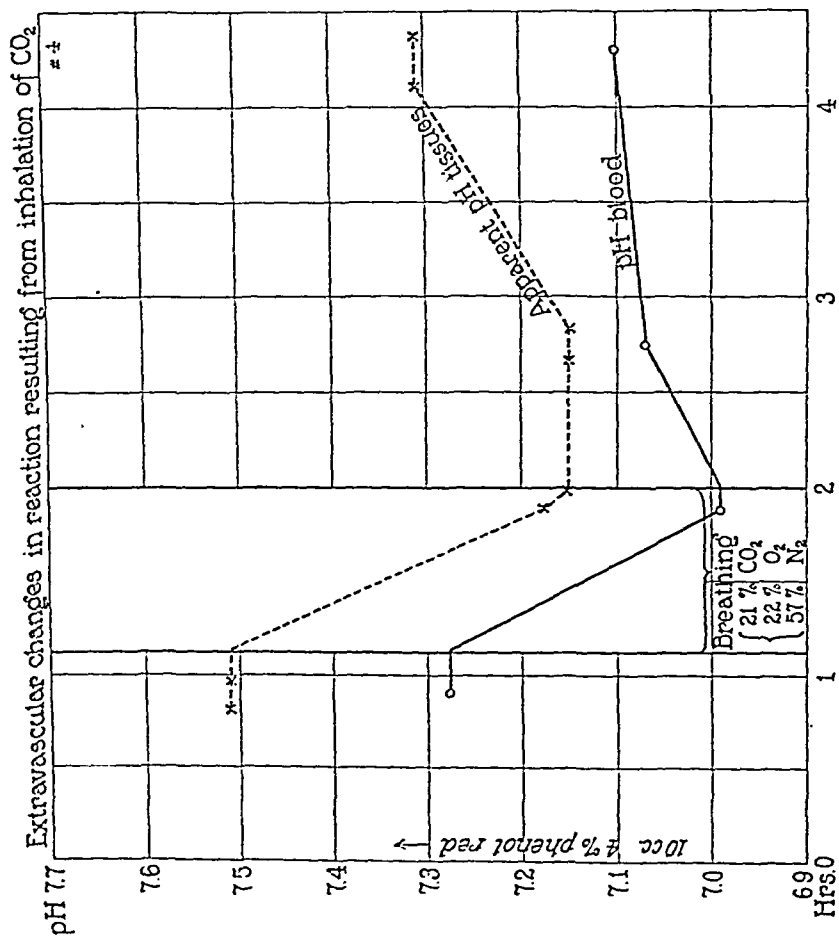
CHART 2.

Extravascular changes in reaction resulting from inhalation of CO_2

is warm.   2.07—*Surface hue* between pH 7.3 and pH 7.4.   Skin folds still much yellower than normal.   Ear cartilage has decolorized too far for appraisal of the hue.   2.18—*Fourth blood specimens taken.*   2.24—*Surface hue* by wedge between pH 7.3 and pH 7.4.   2.25—*Surface hue* between *light jasper red* and *old rose.* Skin fold still is yellower than "normal."   Animal has not stirred at all.   5.00— Color still a medium light pink.   Animal deeply unconscious.   Observations discontinued.   50 cc. of warm water was given by gavage and next day the rabbit was in excellent condition.

The injection of urethane into the peritoneal cavity resulted in a deeper anesthesia than when the same dose was given subcutaneously (Experiments 2 and 3).   The rabbit never moved during the observations, and the staining persisted for an unusually long period.   But more important was the persistence of acidosis after the mask had been taken off (Chart 2).   Comment has already been made (4) on the action of urethane to lessen or prevent the compensatory respiratory response that is usually called forth by an acidosis.   As result of this occurrence the acid products of metabolism tend to heap up within the organism to a greater extent than would follow merely from the introduction of acid from without.   In the present case the extravascular acidosis indicated by the surface hue was not unusually marked; and hence one can scarcely attribute its persistence to an unusual accumulation of acid metabolites.   Doubtless the depression of the respiratory activities persisted after the mask had been taken off.

As a check upon the findings observations were now made in the absence of a general anesthetic.

Experiment 5.—Male rabbit No. 5 of 1500 gm. 10.25–30 a.m.—10 cc. of phenol red given into an ear vein.   The animal at once began to color up rapidly and evenly.   It was placed on the warm pad and a femoral vein bared with the aid of novocaine.   11.02—*Surface hue* slightly less purple than *eugenia red;* by wedge method at pH 7.6.   11.05—*First blood specimens taken.*   The animal is quiet. 11.10—Skin flaps are pink, with the slightest yellow admixture.   The cartilage of the ear is deep pink.   11.14—*Surface hue* by wedge still at pH 7.6.

11.15—*Inhalations begun.*   The breathing at once became rapid and stormy but the animal did not struggle then or later.   The respiratory rate rose abruptly from 80 to 110 but gradually fell again, reaching 78 at the end of the inhalation period.   Throughout it the breathing was greatly exaggerated.

11.17—*Surface hue* rapidly losing its purple quality.   Some linear razor marks heretofore not visible are now rendered suddenly prominent by an orange border

Extravascular changes in reaction resulting from inhalation of CO_2

#5

#6

D. R. DRURY, W. W. BEATTIE, AND PEYTON ROUS

3cc. phenol red i.v. → → "

Breathing
{ 21% CO_2
{ 22% O_2
{ 57% N_2

10cc. phenol red i.v. →

4cc. phenol red i.v. →

Breathing
{ 40% CO_2
{ 20% O_2
{ 40% N_2

pH 7.7

Hrs 0

x----x Apparent pH tissues
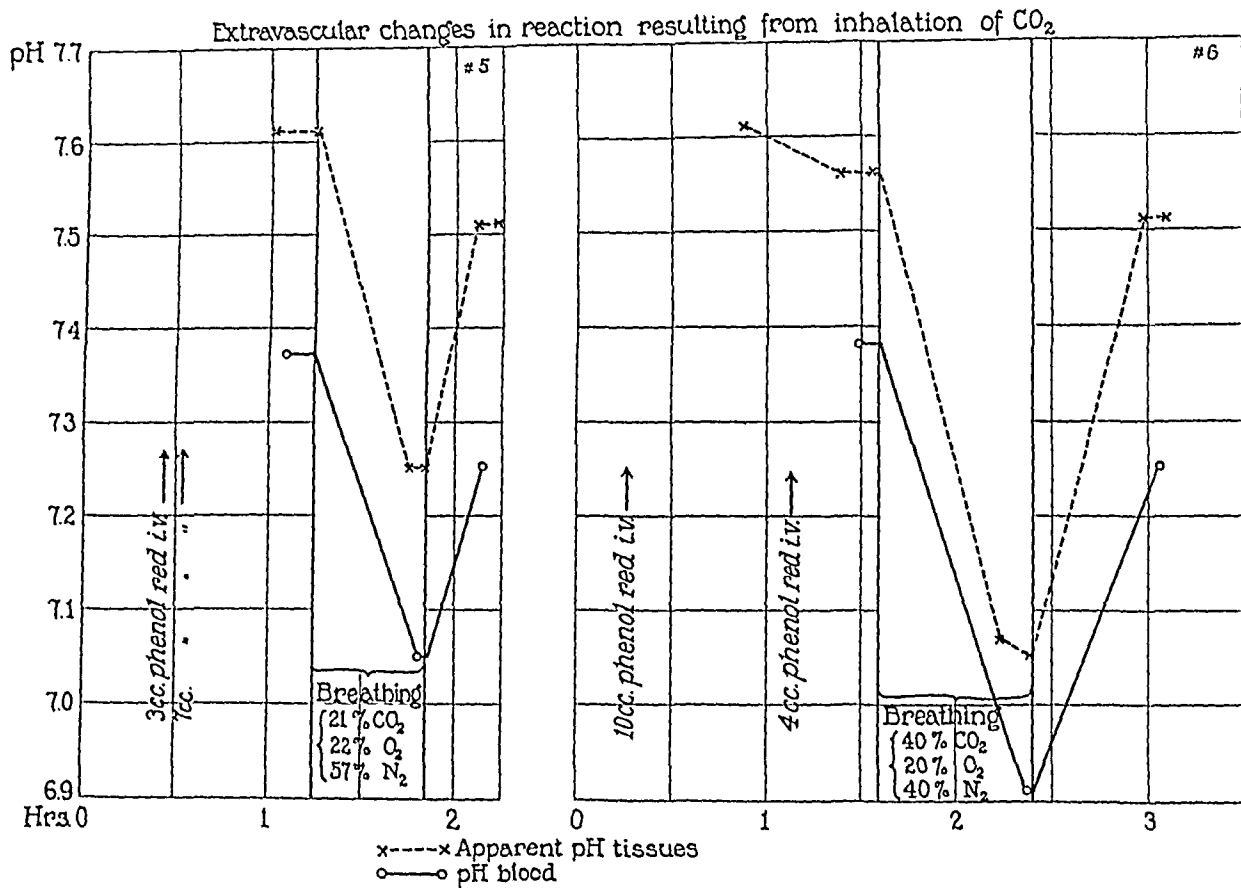o——o pH blood

CHART 3.

49

to either side of them.   *11.20—Surface hue* nearing *jasper red.*   *11.23*—There is a pronounced spotting over the abdomen, of many orange-red patches about 1 cm. in diameter, scattered on a more ruddy background.   The veins are nowhere turgid.   *11.25*—The patching has disappeared and the acidotic hue is confluent save for a broad streak from ensiform to pubis which is still purply red.   The streak corresponds with the region where a poorly vascularized aponeurosis underlies the skin.   The skin flaps are a marked orange-yellow and so too is the ear cartilage.   The circulation in the ear continues excellent.   *11.29—Surface hue* between *coral red* and *light coral red.*   The inside of the ear, where a bloodless cartilage is but thinly overlain with skin, is no longer pink by reflected light but yellow.   The scleral conjunctiva has turned ruddy orange.   There is an excellent conjunctival reflex and the animal appears conscious.   *11.38—Surface hue* slightly lighter than *coral red;* the median streak of more purply red has almost completely merged in the general hue.   Skin flaps are clear orange-yellow; ear cartilage orange-yellow.   *11.44—Surface hue* at pH 7.3 by wedge method.   *11.49— Second blood specimens taken.*   *11.50—Surface hue* at pH 7.3.

11.50—Mask removed.   Immediately the respirations became even more ample than before and the animal appeared excited.   The superficial veins over the abdomen and chest became greatly engorged even in the finer ramifications.

11.52—Surface hue redder and conjunctiva has become pink again; but skin flaps are still orange-yellow.   *11.54*—Animal has now quieted and so too have its respirations.   *11.57—Surface hue* between *eugenia red* and *old rose.*   The venous congestion continues.   *11.59*—The skin flaps have returned to the usual pink with faintest admixture of yellow; the ear cartilage is much pinker.   *12.06— Surface hue* as at 11.57; by wedge method at pH 7.5.   *12.08—Third blood specimens taken.*   *12.15*—The animal has struggled at intervals but remains quiet during repair of the groin incision.   The veins over chest and abdomen are still notably congested.   *12.17*—Circulation in the ears is greatly cut down, and the cartilage is still somewhat yellow both by transmitted and reflected light.   *2.40*— Animal in excellent condition, very light pink.   *3.05*—Animal decolorized except for the ears which show a light pink cartilage.   The superficial veins are still engorged.   Observations discontinued.   The animal was let up, when it behaved normally.

The results of this experiment (Chart 3) corresponded in general with those of the preceding ones.   The tissue acidosis did not become so great, however, and recovery from it was more prompt, though not quite complete when the observations were terminated.

The Acidosis on Breathing 37 Per Cent of CO_2.

During the latter part of the inhalation periods of the foregoing experiments the surface hue changed but little.   It was evident that the

limits had been reached of the extravascular acidosis that could be induced with 21 per cent CO_2. In order to obtain more marked changes resort was had to a mixture containing 37 per cent of this gas.

Experiment 6.—Male rabbit No. 6 of 1500 gm. No general anesthetic. At 10.16 a.m. 10 cc. of phenol red was given into an ear vein and a little later the animal was placed on the warm pad and a femoral vein bared with the aid of novocaine locally. 10.52—*Surface hue* slightly less purple than *eugenia red;* by wedge method at pH 7.6. 11.08—Beginning to decolorize, so 4 cc. more of phenol red given into same vein. 11.23—*Surface hue* slightly yellower than at 10.52; by wedge method between pH 7.5 and pH 7.6. 11.30—*First blood specimens taken.* 11.33—*Surface hue* slightly above pH 7.5. Skin flaps are pink with a trace of yellow, and ear cartilage also. The circulation in the ear is good.

11.35—*Mask on and inhalations begun.* The animal held its breath for about half a minute, then struggled, soon became quiet, and began to breathe regularly and very deeply. During the inhalation period the respiratory rate gradually lessened from 68 to 42 per minute.

11.38—*Surface hue* rapidly becoming yellower, nearing *jasper red* except along midline of abdomen, where as yet there is little change. 11.40—Ear cartilage much yellower. 11.41—Skin flaps already ruddy orange. 11.42—*Surface hue,* generally, *jasper red.* 11.51—Ear cartilage orange-yellow. 11.52—*Surface hue coral red.* 11.53—Animal appears to be completely unconscious; slight rhythmic movements of legs; good conjunctival reflex. 12.00—*Surface hue* about midway between *coral red* and *carnelian red.* 12.01—Skin flaps more orange. 12.05—Bloodless ear cartilage yellow by reflected light. 12.09—*Surface hue carnelian red.* 12.14—*Surface hue* by wedge method at pH 7.1. 12.22—*Second blood specimens taken.* 12.23—*Surface hue* slightly yellower than *carnelian red;* by wedge method pH 7.1 –.

After 12.00 n. the accessory muscles of the neck were called into play during the respirations. By 12.18 these were labored and less extensive. For fear that a gradual failure of ventilation might ensue and complicate the findings the mask was removed at 12.24. At that time there were 42 breaths to the minute. The color of the animal was still changing toward orange. Immediately that the mask was taken off the respirations became much shallower and the rate rose to 82 per minute. The rabbit, previously unconscious to all appearance, raised its head at 12.26 and struggled at 12.27. The ruddy color was now rapidly coming back.

12.28—*Surface hue* approaching *coral red.* 132 respirations per minute, shallow. 12.33—Rabbit quiet; 100 respirations per minute. *Surface hue* here *jasper red,* there *coral red.* 12.36—Conscious but quiet. 12.43—*Surface hue* only slightly yellower than *old rose.* 12.47—Ear cartilage still definitely yellower than ordinary. 12.48—Skin flaps have the normal pink hue. 12.58—*Surface hue* is *old rose;* by wedge method at pH 7.5. 1.03—*Third blood specimens taken.* 1.05—*Surface hue* by wedge, pH 7.5. Experiment discontinued. During its course the rabbit had

lost somewhat less than 5 cc. of blood by a slow escape from the femoral vein. When seen next morning it was in excellent condition.

The effect of breathing an atmosphere containing 37 per cent CO_2 with about the ordinary quantity of oxygen was to render the animal unconscious, and to induce a progressive acidosis (Chart 3). Toward the end of the inhalation period there were signs of respiratory failure; and complications from this source would doubtless have occurred had the mask not been taken off. The extravascular acidosis induced was more considerable than with 21 per cent CO_2 and apparently its limits were not reached. The acidosis of the blood became as pronounced as is ordinarily compatible with life, according to other investigators. Recovery was prompt but not quite complete during the brief period of observation.

Effects of Overventilation.

As a corollary the influence upon the tissue reaction of overbreathing was studied. It was already known that flaps of living and well vascularized connective tissue become more alkaline when exposed to air (5). So too does the peritoneal lining. With overbreathing sufficient to reduce considerably the carbon dioxide tension of the blood one would expect some change in the extravascular reaction. Such a change was obtained, a definite but not a marked one as would follow from the fact that the induced blood alkalosis was but slight. Six experiments were performed, upon rabbits.

Under general anesthesia, brought about with urethane in all save one instance, the animals were tracheotomized and a limb of a T-tube passed down nearly to the bronchial bifurcation and tied in place. In the exceptional case ether was used during the tracheotomy and the lips of the incision were swabbed with novocaine prior to discontinuance of the general anesthesia, at the time when the experiment proper was begun. The tube that formed the staff of the T was connected with the house suction in some instances and in others left open, while the free limb was connected with a motor-run mechanism devised by Dr. F. L. Gates, whereby air, separately warmed and moistened, was blown continuously or rhythmically into the lungs. The chest of some of the urethanized animals was opened by a bloodless incision down the middle of the sternum and a screw retractor inserted to expose the lungs. The blood specimens were taken from a femoral vein, and the surface hue appraised by the wedge method as usual.

Blowing air continuously for half an hour through a catheter with its opening down almost as far as the bifurcation of the bronchi was without effect on the surface hue of the animal stained with phenol red; and when the stream of air was cut off no apnea ensued. Filling and emptying the lungs by the alternate blowing in of air, and suction upon the tracheotomy tube with the house vacuum resulted in well defined changes but great care had to be taken to control the pressure relations, else pulmonary hemorrhage ensued. The best results were obtained in animals with relatively flexible thoracic walls which permitted of a large expansion and deflation of the lungs. It was found that when the chest was opened the animal developed the signs of a marked outlying acidosis despite the existence of an overventilation as proven by the apnea that ensued when the artificial respiration was stopped. In our opinion this acidosis resulted from a peripheral vascular constriction, secondary to and compensatory for, an interference with the circulation which was in turn traceable to an embarrassment of the heart. This last organ,—no longer provided with its usual orientation and supports,—collapsed upon itself at each deflation of the lungs.

The alterations in the reaction of the blood and tissues were never great; and the surface hue was more difficult to appraise in terms of pH than was the case in the acidosis experiments, because the colors were well toward the alkaline side of the range of phenol red. The colors could not always be recorded precisely in terms of Ridgway's nomenclature because the values associated with alkalosis differed somewhat from his scales. Two protocols will be given.

Experiment 7.—(*a*) Male rabbit No. 7 of 2075 gm. given 4 cc. of 50 per cent urethane into the peritoneal cavity at 12.02 and 10 cc. of phenol red into an ear vein at 1.08. A few whiffs of ether were administered during the tracheotomy, which had been accomplished by 1.27 p.m. 1.37—*Surface hue* between *eugenia red* and *jasper red* but deeper than either; by wedge method pH 7.5 —. Skin flaps pink, with faintest yellow admixture.

1.40.—*Artificial respiration by pressure and suction begun.* The suction was sufficient to cause marked depression at the costochrondral junctions and the normal respiratory rhythm was easily overcome. 1.46—On intermitting briefly the artificial respiration an apnea ensued lasting 45 seconds. 2.00—*Surface hue* deeper than *eugenia red;* by wedge pH 7.6+. 2.01—Skin flaps deep purply red, no yellow.

2.23—*Discontinued artificial respirations.* Surface hue at this time just above pH 7.6 by wedge. Apnea for 70 seconds; then resumption of breathing at 44 respirations per minute. During the suction and compression the rate had varied between 28 and 32. 2.27—*Surface hue* returning toward *eugenia red.* 2.39—*Surface hue* between *eugenia red* and *jasper red.* 2.55—*Surface hue* less purple and slightly lighter than *eugenia red.* Skin flaps once again show a tinge of yellow in the prevailing pink.

(*b*) 3.00 p.m. of same day—*Surface hue* by wedge pH 7.5. 3.01—*First blood specimens taken.* 3.05—*Surface hue* by wedge pH 7.5 —. Skin flaps colored as before. Respirations 42 per minute.

Chart 4.
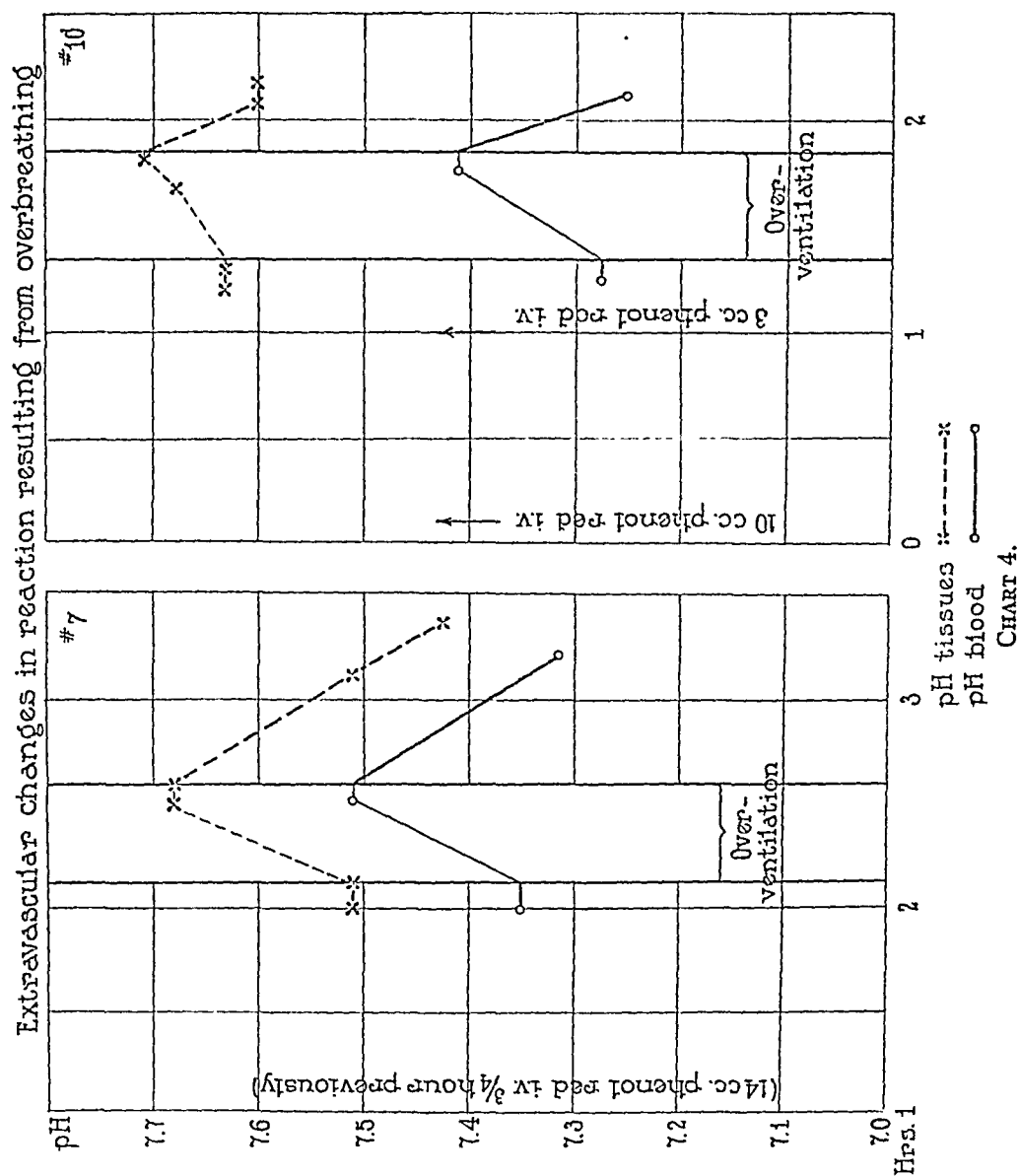
Artificial respirations begun at 32 per minute. 3.08—Animal decolorizing. *Surface hue* only slightly darker than *old rose*. 3.17—*Surface hue* now much darker and purpler than *eugenia red*. 3.31—Decolorization proceeds. *Surface hue* lighter than *eugenia red;* by wedge midway between pH 7.6 and 7.7. 3.32—*Second blood specimens taken.* 3.34—*Surface hue* by wedge between pH 7.6 and 7.7.

3.35—*Artificial respiration stopped.* Apnea of 50 seconds. 3.37—Respirations 146 per minute, *surface hue* much less purple. 3.59—Respirations have gradually become slower, now 54 per minute. *Surface hue old rose,* by wedge pH 7.5. 4.13—*Third blood specimens taken.* 4.20—*Surface hue alizarin pink,* by wedge pH 7.4+.

At 4.26 the animal was killed with ether and autopsied. It showed two localized pulmonary hemorrhages, 0.5 and 1.5 cm. in diameter respectively, one in each lower lobe.

Experiment 8.—Male rabbit No. 10 of 1600 gm. Given 1.3 cc. of urethane into tissues of back of neck at 12.30; 1 cc. more into peritoneal cavity at 2.00; and a few whiffs of ether during the tracheotomy, which was accomplished by 2.30. Between 2.21 and 2.25 10 cc. of phenol red was injected into an ear vein. The vital staining was even and deep, but owing to time lost in some accessory observations it was necessary to give 3 cc. more of the phthalein, at 3.30, to compensate for the decolorization that was taking place. 3.43—*Surface hue* slightly lighter than *eugenia red*, at pH 7.6 by wedge. 26 respirations per minute. 3.47—*First blood specimens taken.* 3.48—*Surface hue* again, pH 7.6.

3.50—*Artificial respirations begun* at rate of 28 per minute by alternating pressure and suction. 3.58—The blood in the superficial veins appears much purpler, so that these are more clearly seen. (The like observation was made in other experiments not reported here in detail.) 4.11—*Surface hue* by wedge between pH 7.6 and pH 7.7. 4.16—*Second blood specimens taken.* 4.19—*Surface hue* purpler and lighter than *eugenia red*, by wedge pH 7.7 −.

Artificial respirations stopped. Apnea for only 35 seconds. Thereafter the respirations were at first rapid, 140 per minute, and then gradually slowed, to 82 per minute. 4.24—*Surface hue* lighter and only faintly purpler than *eugenia red*. 4.34—*Surface hue* slightly lighter and faintly purpler than *old rose;* by wedge method pH 7.6 −. 4.38—*Third blood specimens taken.* 4.40—*Surface hue* by wedge pH 7.6 −. Respirations 86 per minute. Observations discontinued. The rabbit was chloroformed for examination, on the same day. No lesions were found other than a liver coccidiosis.

Changes in the color of the connective tissue and cartilage could not at any time be made out.

While the alterations in reaction produced in these experiments (Chart 4) were not great they were sufficient for the purpose in hand, namely to determine whether an extravascular alkalosis is linked with

the blood alkalosis induced by overbreathing.   Such is the case, the alterations in the blood reaction being closely paralleled by similar extravascular ones, as attested by the surface hue.   In one of the two experiments the purple hue of skin flaps compressed between slides and viewed by transmitted light indicated the development of a more alkaline reaction of the connective tissue.   No such change was to be seen in the ear cartilage.

DISCUSSION.

The experiments show that under the circumstances of acidosis due to the inhalation of carbon dioxide, and of alkalosis consequent on a blowing off of the gas, changes occur in the extravascular reaction which closely parallel those in the blood.   Without doubt the extravascular changes largely involve the interstitial fluids, but during the acidosis concomitant alterations take place as well in the connective tissue and cartilage, the matrix tissues so readily rendered acid by the intravenous injection of acid solutions.   During the alkalosis we noted connective tissue changes only, but this is not surprising since it was slight in degree and of brief duration.   In our experience alterations in the reaction of cartilage follow rather tardily upon those of the connective tissue (*vide* Experiment 3); and this is especially true of the cartilage in the ear of the rabbit, which is almost avascular in the regions best suited to inspection.   No observations were made upon the deep-lying organs; but one would expect from the results in rats given hydrochloric acid (6) that under the circumstances of acidosis due to carbonic acid the tendons would also become acid.   Whether the reaction would also change in the liver, pancreas, and lymph nodes, parenchymal organs unaffected apparently by even the most extreme hydrochloric acid acidosis, is an interesting question.   Quite possibly this would happen.   For carbon dioxide manifests abilities to penetrate living tissue far beyond those of other acids (7).

An extravascular acidosis began to develop practically at once on the inhalation of the carbon dioxide, whereas it did not appear until a large quantity of hydrochloric acid had been run into the blood.   This was to have been expected since the introduction of hydrochloric acid is compensated for in large measure by the elimination of carbon dioxide through the lungs, a possibility excluded when the gas is

being taken into the body by the pulmonary route. But over and above this difference one may attribute the prompt development of the extravascular CO_2 acidosis to the known ability of the gas to penetrate tissues rapidly (7). Evidence of such penetration was clearly to be seen in the course of our experiments. Where the circulation was unusually good, as for example about the almost imperceptible abrasions due to shaving (Experiment 5), the acidosis developed soonest; and where the vascularization was scanty, as along the line between ensiform and symphysis (Experiments 4 and 5) there it was seen last. The patching with color sometimes witnessed during the course of the inhalations (Experiments 3 and 5), and resembling that of outlying acidosis, was in reality consequent on a diametrically opposite state of affairs, those parts becoming most rapidly acid, and in consequence standing out in orange-red against a red background, which were most accessible to the acidotic blood, not least so.

The continuance of a carbon dioxide acidosis would doubtless lead to some accumulation of acid metabolites here and there in the body; but during the brief period of our experiments little evidence of such an event was obtained. The reaction of the blood and tissues swiftly changed for the better when the inhalations were discontinued, in significant contrast to the persistence of acidosis when hydrochloric acid had been administered. True, the reaction did not quite return to the normal even under the best of conditions (Experiments 5 and 6), and a compensated acidosis may well have been present and have endured for some time. To determine the actual case was no part of our work which had for sole aim the immediate influence upon the tissue reaction of alterations in the CO_2 content of the blood.

The normal reaction of the blood, as obtained from the femoral vein in the present experiments, varied from about pH 7.25 to slightly less than pH 7.4, whereas that from an ear vein, as noted in our preceding work (8), ranged from just below pH 7.4 to nearly pH 7.5. This very considerable discrepancy between the reaction of the two sets of specimens read in precisely the same way and by the same observer was a consistent finding. The fact may be recalled in this connection that the blood had come from very different regions. Since our object was to follow the relative, not the actual, variations

in intravascular and extravascular reaction the problem thus raised will not be discussed.

Hawkins has shown that urethane anesthesia results in an alkalosis (9); yet there can be no doubt that under uncomplicated conditions it tends to prolong the acidosis resulting from the inhalation of carbon dioxide (Experiment 4, Chart 2), as also that induced by hydrochloric acid. There is evidence that this comes about through a change in the respiratory center (10).

SUMMARY.

Breathing an atmosphere that contains the normal amount of oxygen but a large excess of carbon dioxide results in a tissue acidosis as well as one of the blood. The extravascular changes in reaction take place with far greater speed than when acidosis is induced with hydrochloric acid, and they do not persist as in the case of this latter but swiftly disappear when the animal breathes ordinary air once again. The changes parallel closely in magnitude and time those occurring in the blood. The same matrix tissues are rendered acidotic as when hydrochloric acid is administered.

The blood alkalosis that results from a blowing off of carbon dioxide is accompanied by an extravascular alkalosis. Under the circumstances of our experiments the connective tissue became more alkaline than ordinary but no change was noted in the cartilage, a fact to be explained by the slight degree of the alkalosis and its brief duration.

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THE QUANTITY OF CHOLESTEROL IN THE BLOOD SERUM OF THE GUINEA PIG AS AN INHERITED CHARACTER; ITS RELATION TO NATURAL RESISTANCE TO TUBERCULOSIS, AND TO TUBERCULOSIS INFECTION.

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The literature offers certain suggestions that changes in the distribution of cholesterol may be a feature of the pathology of tuberculosis.

Caldwell (1) analyzing tuberculous bovine lymph gland and liver tissue found two to three times the cholesterol content normal for these tissues. The percentage increase varied with the degree of caseation. Jaffé and Levinson (10) observed a marked increase in optically active fat in the tubercles of rabbits fed cholesterol in oil solution. Thus it would seem that there may be an increase of cholesterol at tuberculous foci and that the content of this substance may be carried still higher by administration of the substance.

Bacmeister and Henes (4) found either normal or increased values for blood cholesterol·in tuberculous patients who were doing well and in good condition, while low figures were obtained in the stage of cachexia. Chauffard, Laroche, and Grigaut (5) obtained similar results. Weltmann (6) found normal cholesterol figures in early tuberculosis, but low ones in advanced, the amount falling rapidly in acute cases. Henning (7) obtained practically normal plasma cholesterol values when using the non-saponification method of cholesterol determination, while with the saponification method he obtained results uniformly much lower. He considered that this indicated the presence, in the blood of tuberculous individuals, of some substance other than cholesterol which gives the cholesterol color reaction but which is sensitive to alkali. Sweany, Weathers, and McCluskey (8) failed to find such a marked difference when using these two methods although the saponification method gave slightly the lower blood cholesterol values. This difference was not more marked, however, than have been noted (9) on a comparison of the two methods in conditions other than tuberculosis. They found that blood cholesterol values were highest in tuberculous patients who were doing well and low in patients doing poorly and losing ground.

Jaffé and Levinson (10), using rabbits infected with bovine type tubercle bacilli, found that following infection the blood cholesterol began to rise from a normal of 80 and 90 mg. per cent to 115 to 130 mg. per cent. In one animal this high value was maintained, while in the other animal it dropped before death to almost the original value.

The authors cited agree that the blood cholesterol is decreased in the more advanced stages of tuberculosis. In the earlier stages normal or increased values have usually been found. This refers to human beings and to rabbits. I have found no reference to determinations of blood cholesterol on either the normal or tuberculous guinea pig.

The inbred guinea pigs as used by Wright and Lewis (2), and Lewis and Loomis (3), are being maintained. In view of the variations in natural resistance to tuberculosis existing among inbred families of guinea pigs, it seemed not impossible that these animals might show some differences referable to their cholesterol metabolism. Dr. Lewis has kindly put his stock at my disposal for determinations of the cholesterol of the blood serum.

These experiments were therefore planned to determine the influence, if any, of an infection with the tubercle bacillus of bovine type on the cholesterol content of the blood of the guinea pig; and also to determine the normal cholesterol content of the blood of the available inbred guinea pigs, with particular reference to any familial variation.

<div align="center">EXPERIMENTAL.</div>

It was found that there was a large individual variation in blood cholesterol, especially pronounced with the females. Observations were therefore confined to large groups of males.

Sixteen male guinea pigs from inbred Family 35 and the same number from inbred Family 13 were bled 1 cc. from the heart, and the 16 cc. of blood thus obtained was pooled and allowed to clot in the refrigerator. The clot was later broken up and the specimens centrifuged to obtain the serum. 3 cc. quantities were used in the determinations, and Bloor's method (9) was employed throughout with the modification that the amounts of alcohol-ether extractive were doubled because of the low serum cholesterol values in guinea pig blood. By using 20 cc. of alcohol-ether extract instead of 10 cc., colors readable in the colorimeter could be obtained. The cholesterol value obtained in this way had of course to be

TABLE I.

	Uninfected Animals	

Bled Feb. 4, 1926.   Diet: Hay, oats, and mangels

| Family No. | Serum cholesterol | |
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	59.5	58
	58.6	
	55.8	
35	64.6	65
	64.5	
	64.5	

Feb. 5, 1926, diet changed to hay and soaked oats daily, cabbage once a week
Bled Feb. 9, 1926

13	56	56
	57	
	57	
	55	
35	69	69
	66	
	71	
	69	

Bled Feb. 15, 1926

13	61	60
	60	
	60	
	59	
35	67	68
	68	
	67	

Feb. 18, 1926, infected with 1/10 mg. of a culture of bovine tubercle bacilli (Bov. 14), intraperitoneally

TABLE I—*Continued.*

Infected Animals

Bled Feb. 23, 1926

Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	58 58	58
35	63 66	64.5

Bled Mar. 1, 1926

13	57 56 57 57	57
35	75 73 68 68	71

Bled Mar. 8, 1926

13	60 59 60 58	59
35	65 64 67 68	66

Bled Mar. 18, 1926

13	59 59 60 59	59
35	67 67 68 68.5	68

TABLE I—*Concluded.*

| | *Average for Total Experiment* | |
	Family 35	Family 13
Before infection............	67 mg. per cent	58 mg. per cent
After "	67 " " "	58 " " "

divided by two.   Each specimen of serum was run in duplicate and the extractives in turn, were run in duplicate in most cases.

The animals were bled three times at intervals of 5 or 6 days.   They were then inoculated intraperitoneally with a virulent culture of the tubercle bacillus of bovine type and again bled at intervals of 7 to 10 days after the infection had been established.   Animals killed accidentally while bleeding were replaced, until the time of inoculation.
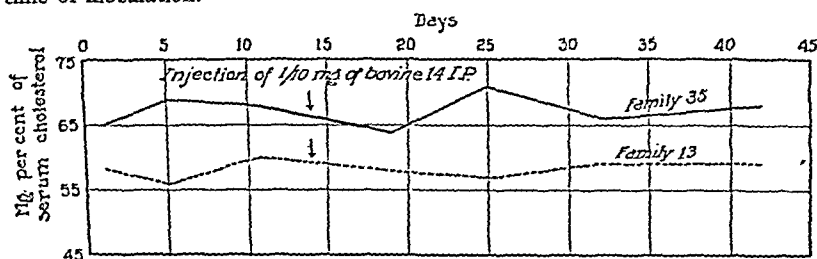

CHART 1.

The results of the serum cholesterol determinations are given in Table I, and graphically in Chart 1.

From these results it is evident that a tuberculous infection in guinea pigs produces no marked change in the serum cholesterol values.   There was no rise at the onset of infection and no terminal fall was noted, although at the time of the last bleeding the animals were losing weight rapidly and had begun dying of tuberculosis. The animals in Family 35 showed uniformly higher serum cholesterol values than did the animals in Family 13.   For reasons apart from present considerations, the diet was changed after the first bleeding of the period prior to infection and the diet then established was maintained throughout.   The change was evidently without effect on the cholesterol content of the serum.

A further series of examinations was now conducted involving guinea

pigs from each of the four inbred families whose reactions to tuberculous infection are known.   In this case a change of diet was also introduced following the first bleeding and 2 weeks before the second.

TABLE II.

Bled Mar. 30, 1926. Diet: Timothy hay, oats, and mangels		
Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
13	57 57	57
35	66 66	66
32	64 61	62.5
2	49 51	50
Apr. 1, 1926, diet changed to alfalfa hay and oats, cabbage once a week Bled Apr. 13, 1926		
13	55 55	55
35	61 69	65
32	66 66	66
2	52 53	52 5

Since the previous results had made it apparent that tuberculous infection produced no changes in the serum cholesterol values in guinea pigs, the determinations this time were only on normal, uninfected animals.   Eight male guinea pigs of about the same weight and age from each family were used for this experiment and the blood was handled in the same way as before.   The results are given in Table II.

In this series, guinea pigs of Family 35 again showed a uniformly higher serum cholesterol content than those from Family 13 and the values obtained for each family are comparable to those obtained in the first experiment. Guinea pigs of Family 32 have values very close to those for Family 35, while the figures obtained for Family 2 are lower than those found for Family 13. On the basis of serum cholesterol the order of the families from high to low is 35, 32, 13, 2. The substitution of alfalfa for timothy hay in the diet had no apparent effect on serum cholesterol concentration.

TABLE III.

No. of guinea pig	Family	Serum cholesterol	
		Separate readings in mg. per cent	Average for families in mg. per cent
1	13	50	50
2		48	
3		50	
4		52	
5	35	63	59.6
6		58	
7		59.5	
8		58	

In order to determine how wide might be the individual variations among the animals of Families 13 and 35, serum cholesterol determinations were run on four male guinea pigs from each of the two families. 8 cc. of blood was drawn from the heart of each animal and 3 cc. of serum was used in the determination, following the same method as in the previous analyses. The results are recorded in Table III.

In this series of determinations the serum cholesterol values ran uniformly lower than in the preceding series. This may have been due to the fact that early in May, 2 weeks prior to the bleedings, the animals were put on a succulent diet consisting of freshly cut clover and alfalfa. Practically the same differences prevail between the two families although on a lower level. In the first experiment the average values obtained were, for Family 35, 67 mg. per cent, and for

Family 13, 58 mg. per cent, a difference of 9 mg. per cent. In this series the difference noted in the average of the values is 9.6 mg. per cent. It is also to be noted that the individual variation appears to be from 4 to 5 mg. per cent. Comparison with the tables of the earlier experiments shows that this is within the limits of variation shown by the separate readings on the same blood sample and indicates that the individual differences cannot, with the technic employed, be regarded as significant.

DISCUSSION.

The results obtained have interest in several directions. It is clear that the progress of tuberculosis in the guinea pig is not marked by changes in serum cholesterol. This is in suggestive contrast to the observations on the human disease and to the experiments of Jaffé and Levinson on the rabbit. The differences may depend on a different relationship due to species, but it is also possible that the changes found by previous observers, which have been changes in the cholesterol content of the whole blood in most cases, may be due to changes in the corpuscles.

Any changes in diet under controlled conditions, as in Experiments 1 and 2, were without appreciable influence on the serum cholesterol. This was to have been expected since the cholesterol and phytosterol concentrations of the interchanged foods were probably not very different and the change being from one dry food to another the water exchange was probably not affected. The lower values obtained in the third series of observations might well be due to the succulent diet being fed at the time, or to other seasonal changes.

The familial differences in serum cholesterol have been consistent and are of wide interest. The families of guinea pigs used had been strictly inbred for many generations and may be regarded as homozygous. Differences between them in other respects are pronounced and constant. They relate to such characters as color, conformation, size, and fertility, and have been fully discussed by Wright (11). The concentration of serum cholesterol appears to be a well defined character peculiar to the family, and hence, considering the nature of the animal material, one controlled by the inheritance. We are familiar

with only one other recorded instance of an inheritable character related to a particular feature of the general metabolism. Benedict (12) has described peculiarities in the purine metabolism of the Dalmatian coach hound which have a similar relationship. It is of importance that our knowledge of similar instances should be multiplied as preliminary to a more definite association of genetics with physiology and pathology.

The families of guinea pigs employed for the present work possess different levels of natural resistance against infection with the tubercle bacillus (2). Wright and Lewis were led by a comparison of results with crosses between the families to assume that the total of differences found must be due to the interrelated operation of three or more separately inherited characters. The question now arises whether the serum cholesterol content is one of the three or more factors predicated by these authors. The order of familial resistance from high to low as found by Wright and Lewis was 35, 2, 32, 13. The order based on the serum cholesterol concentration from high to low is 35, 32, 13, 2. These relations make it plain that the cholesterol concentration cannot be the only factor in determining the resistance to tuberculosis, nor can it be the controlling factor.

SUMMARY.

1. Infection with tubercle bacilli of bovine type produced no alteration in the serum cholesterol content in guinea pigs.

2. Certain controlled variations in diet produced no changes in the serum cholesterol content in guinea pigs.

3. Inbred families of guinea pigs known to manifest differing resistances to tuberculosis gave differing serum cholesterol values, but no direct relationship between the two sets of phenomena could be established.

4. It seems probable that in the guinea pig the cholesterol content of the blood serum is influenced by inherited factors.

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STUDIES IN BLOOD COAGULATION.

I. Certain Characteristics of Coagulation and Their Measurement.

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Plate 1.

In the study of a malignant transplanted tumor of the rabbit (1, 2) it has been observed that animals pursuing a rapidly fatal course of the disease frequently show as a terminal event evidences of an hemorrhagic state, with extravasation of blood into muscles, connective tissue spaces and serous cavities. Blood tests demonstrate prolonged' clotting rate and imperfect clot retraction. In earlier stages, also, of this florid and of milder forms of the tumor are found changes in blood coagulability, less extensive and consistently related to various phases of the tumor growth. The findings are to be reported in Part II of this communication, it being necessary first to consider in detail the characteristics of blood coagulation that were investigated and the means adopted for their evaluation.

In estimating blood coagulability particular attention has to be paid to the circumstances of the technique employed, since they of themselves may influence the readings very considerably. This has been emphasized abundantly by others, referring to the markedly accelerating effect of "tissue juices" upon coagulation (3), the acceleration produced by various foreign substances, metal, glass, etc., coming in contact with the specimen during the test (4), the retardation in clotting rate resulting from periodic or continuous disturbance of the blood necessary in making observations (5), or from exposure to the air, also, the important part played by the temperature at which coagulation takes place (6). While the effects of extraneous factors of this sort may be maintained constant in a given technique and the readings obtained be of significance relative to each other, the

result may be to remove the scale of values so far from the basic rate of coagulation as to obliterate certain abnormalities that occur. For example, cases of hemophilia have been described (7–9) in which the clotting time of blood taken directly from the circulation was greatly extended, while specimens that had touched the tissues in being secured coagulated at normal rates. These were extreme instances but they illustrate the importance of obtaining as nearly as possible a basic clotting rate value; and for the present work where but slight changes in blood coagulability are frequently presented such factors have had to be eliminated from the tests, a condition not satisfied by by any of the procedures known to us. Effort has been made, accordingly, to develop methods with conditions of technique nearly neutral in all regards to coagulation. Indicators were sought for to serve as bases of measurement, phenomena occurring distinctively and spontaneously in blood coagulating at rest, rather than the arbitrary type of standard usually employed, such as certain degrees of firmness, adhesiveness, ductility or plasticity of the clot.

The problem at hand required consideration of two phases of the blood coagulative process, *i.e.*, clot formation and clot retraction. These will be dealt with separately; first, the characteristics of each will be discussed, and then methods of measurement described.
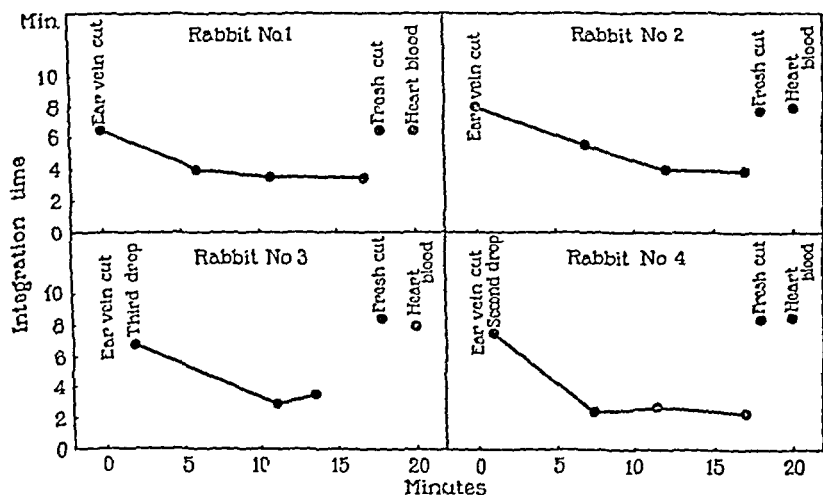
I. Clot Formation.

Experimental Basis.—A drop of freshly shed blood deposited under clear, bland oil and resting upon paraffin remains intact as a slightly flattened sphere and presents an exquisitely smooth and sensitive surface (Fig. 1, No. 1). Observed at room temperature, the sphere of blood remains without change of appearance for about 15 minutes, when, almost imperceptibly at first, retraction of clot begins as a minute dimple or wrinkle at one point or another (Fig. 1, No. 2), or as a warping of the whole specimen. Soon the distortions multiply and extend, and in the space of a minute or so the drop loses completely its symmetry and finish (Fig. 1, No. 3). Further retraction follows and bubbles of serum are extruded (Fig. 1, Nos. 4 and 5).

It is here evident that clot retraction commences in a specimen of blood only after a considerable period has elapsed since shedding, during which time the clot is being formed. Apparently, the fibrin

coagulum must undergo a certain amount of development or maturation before it becomes capable of retraction, and, once this condition has been attained, retraction proceeds rapidly. The duration of this period will be referred to as clot formation time, and, with the above preparation, it will be measured with the onset of retraction as endpoint.

In the rabbit it is not practicable for repeated tests to take specimens of blood directly from the circulation by puncture of large vessels, and section of the ear vein must be employed.

TEXT-FIG. 1.

Technical factors in this procedure which may influence the results are indicated by the following experiment: In each of four rabbits a series of drops from the same wound were tested for clot formation time; also, blood was tested from a fresh cut and from heart puncture. The results are charted in Text-fig. 1. It will be seen that clot formation quickens progressively in blood as it flows from the vein incision, up to a certain limit which may be twice as rapid as that at first. Blood from a fresh cut repeats the first reading obtained, as well as that drawn from the circulation without tissue contact. Thus, under circumstances prohibiting venepuncture, venesection may be used provided the first specimen of blood is taken. Skin puncture alone should not be used, as here the blood flows less rapidly and may be altered from the onset by admixture with "tissue juices."

The temperature at which the specimen coagulates requires control. The effects of temperature change upon the rate of clot formation are shown in Text-

fig. 2 at *A*. Curves from two rabbits are given, in each of which four specimens, taken simultaneously by heart puncture, were examined at different temperatures, ranging from 17–39°C. Clot formation is seen to vary in rate with the temperature, being greatly delayed at the lower levels. The relation, however, is not strictly direct, for the change in rate per degree change in temperature becomes greater as the scale is descended. Body heat (37.5°C.) is the logical condition for the test but this is too high to be maintained readily by the simple means of heat regulation to be described, and 32°C. has been selected. This temperature
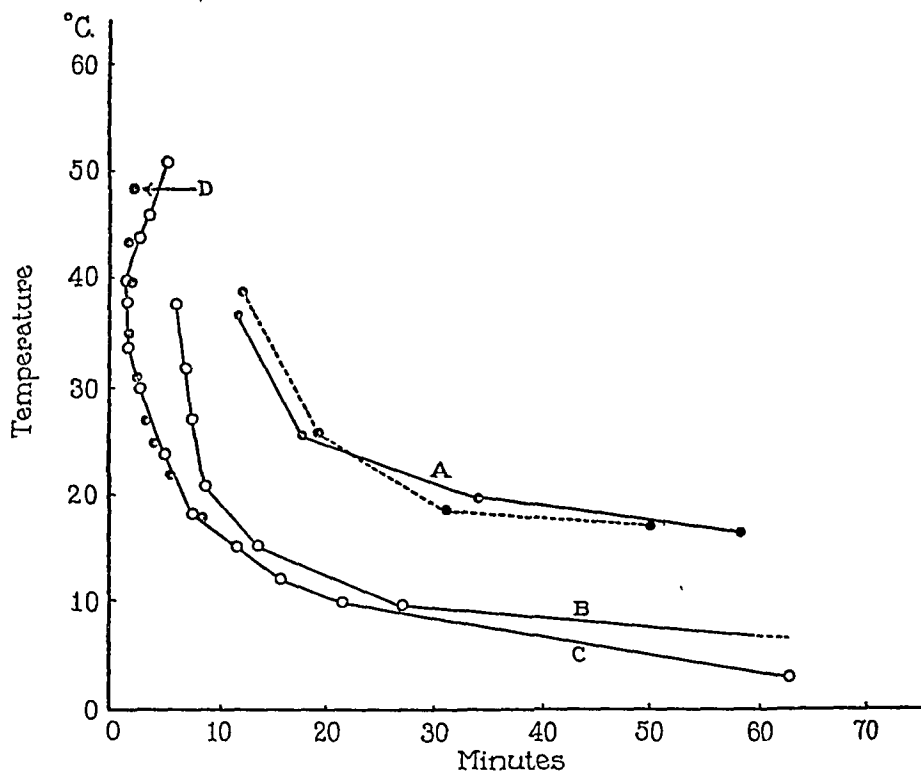
TEXT-FIG. 2.

is sufficiently high that the readings differ but slightly from those at 37.5°C. and, also, are affected inconsiderably by variations in temperature to the extent of 1° or 2° as may occur inadvertently in the technique.

Apparatus.—The articles required are a specimen platform, test-tube and holder, water bath and thermometer (Text-fig. 3). The platform is a thin, concave disc of metal, 1.0 cm. in diameter, supported upon a rod 10 cm. in length. The test-tube is so proportioned as to receive the platform disc readily, is 12 cm. in length and made of clear glass. The test-tube holder is of the type illustrated. A vertical sided jar of about 2 liters capacity serves as water bath.

The parts are assembled for use as follows: The platform disc is coated to at least 1 mm. thickness by dipping in melted paraffin several times, care being taken to preserve the concavity of the upper surface. The tube is filled with mineral oil (Squibb's) to within a few mm. of the top, and into it is dropped the platform, the disc of which will then lie about 1.5 cm. under the oil (Text-fig. 3, A). The tube, grasped in the holder, is now suspended part way in the water bath (at about 45°C.) and 5 minutes time is allowed for the column of oil to reach a stationary temperature (Text-fig. 3, B). The temperature of the layer of oil overlying the platform is then taken, the thermometer bulb first being warmed in the hand. It is desired to obtain in this section of the oil column a heat level of 32–33°C., and the tube
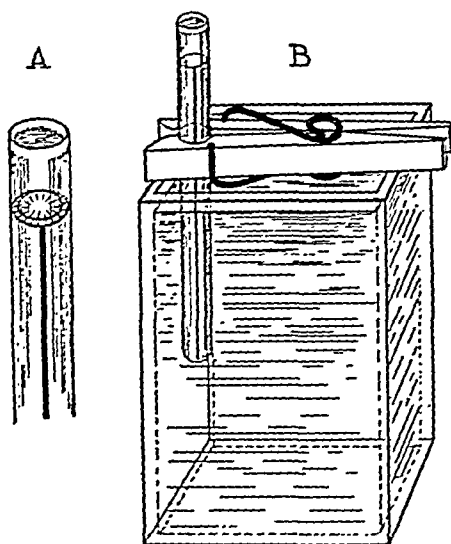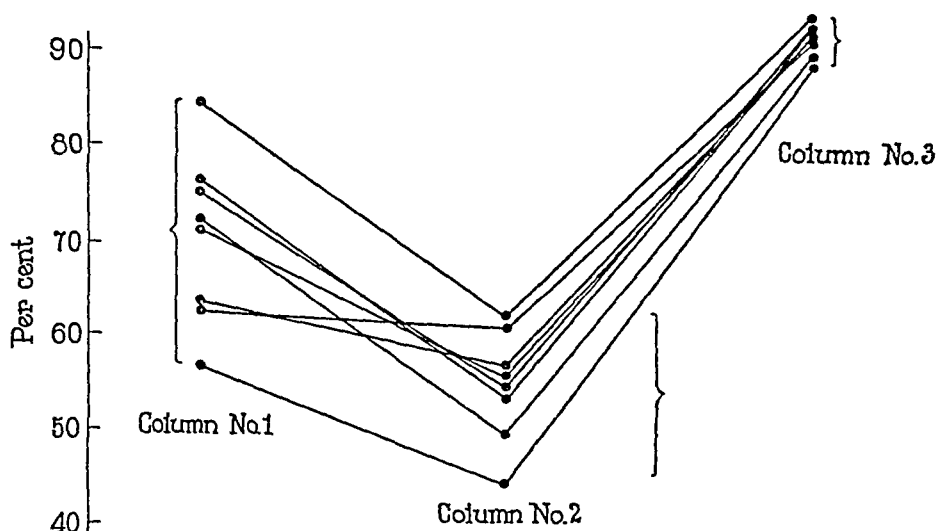

TEXT-FIG. 3.

is further lowered or raised in the bath until the temperature is secured. Once adjusted, it will not fall appreciably during the test.

In dealing with a series of animals a rack of test-tubes and a larger water bath have been used. The thermometer is fixed in one tube with its bulb at the level of the platform discs, and by adjusting the height of the rack in the water, and lowering it slightly from time to time as the water cools, all tubes may be kept at the desired temperature for as long as 3 hours while they are being used.

Method.—A small quantity of blood is drawn with a syringe, and, with the point of the needle immediately under the oil, a single drop about 0.6 cm. in diameter is injected and allowed to rest upon the platform, care being exercised that it does not touch the sides of the tube. The size of the drop is unimportant except as the

larger specimens are more easily read. It is observed closely for the first evidence of clot retraction. Fine indentations in the surface indicating this are best seen by bringing the high light to play on the sides of the drop; and warping is detected from above. The interval of time elapsing between securing the blood and the onset of retraction is recorded.

The blood specimen is obtained in the rabbit usually from the ear vein. The skin surface here is shaved, cleansed and dried, then vessel dilatation is produced by applying warmth underneath (small electric light bulb) and a razor cut is made directly into the vein where it lies beneath the skin. Blood will spurt out, and the first that appears is drawn into a syringe or rubber bulb pipette (medicine dropper) for testing.
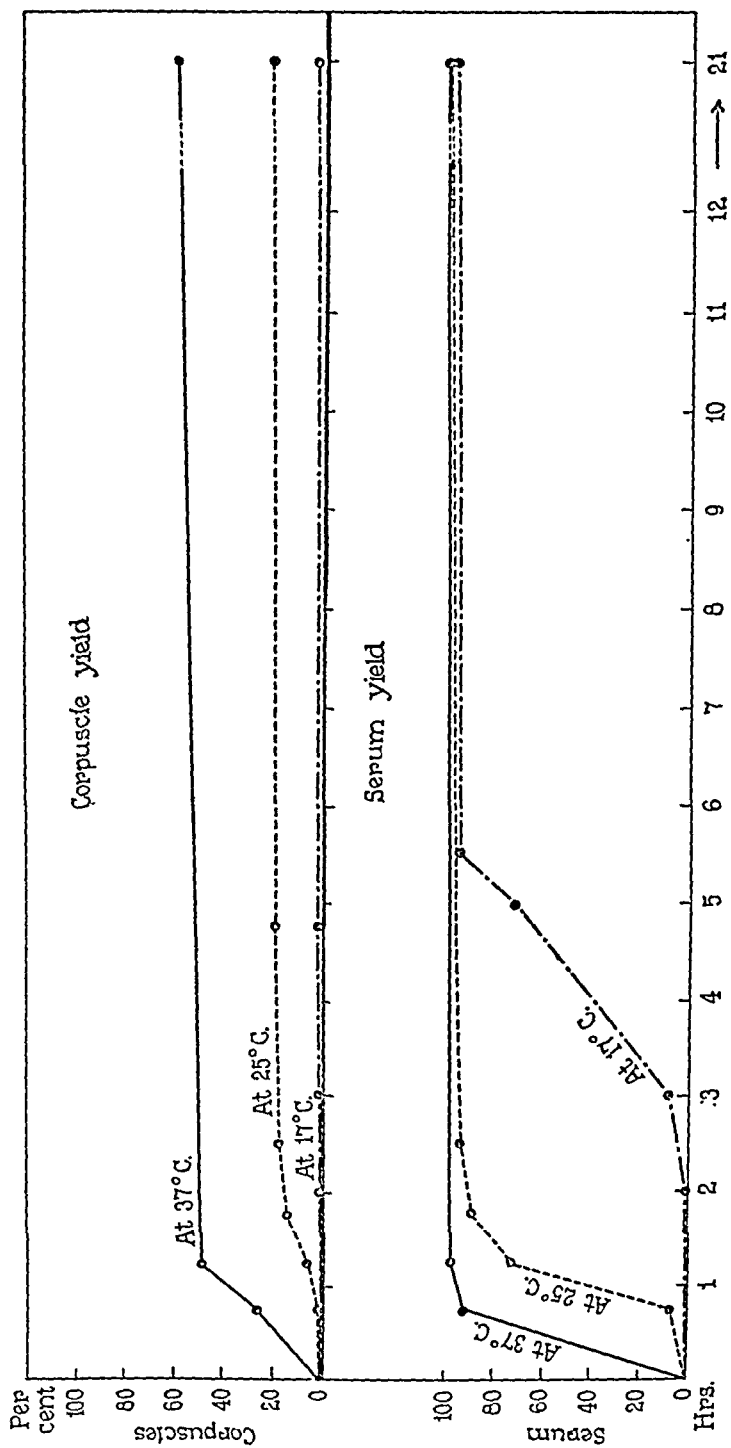

TEXT-FIG. 4.

Results.—70, or one-third, of the number of rabbits whose clot formation time at 32°C. has been examined, were normal animals and gave readings between 4 and 8 minutes, averaging $5\frac{1}{2}$ minutes. In those with various diseases the readings varied between 2 and 36 minutes. Incoagulable blood renders no end-point; the drop remains symmetrical and the corpuscles therein sediment, leaving a supernatant fluid of ground glass opacity.

Corpuscle sedimentation occurs also to a degree in certain blood specimens which go on to coagulation. This is accompanied by a prolonged clot formation time, although the latter is frequently found independently. The significance of sedimentation will be discussed below.

II. Clot Retraction.

Experimental Basis.—Extent of Retraction.—Since blood clot retracts with a loss of substance (serum and an amount of corpuscles), it is possible to study the characteristics of clot retraction quantitatively in terms of this substance. For example, if a known amount of rabbit blood be collected and allowed to coagulate completely, it will be found that the material which exudes from the clot amounts to about 70 per cent of the specimen; or, in a series of normal rabbits, between 55 and 85 per cent (Text-fig. 4, Column 1). If these exudates are then centrifugalized, to separate the corpuscles, and the amounts of serum compared, a lesser range of variation will be found (Text-fig. 4, Column 2). But it is logical to suppose that a given specimen of blood can deliver serum only in amount depending upon the available fluid (plasma) content. If, therefore, estimations of the plasma-corpuscle ratios in the blood of these animals be obtained (hematocrit test) and each of the above serum values be expressed in percentage of the plasma content, the range of results will be reduced to within narrow limits (Text-fig. 4, Column 3). It is evident, accordingly, that the normal extent of retraction of blood clot, expressed in terms of the relative amount of fluid lost in the process, is a fairly constant function.

Rate of Retraction.—The rate with which clot retracts, moreover, can be indicated by taking readings of this sort at intervals during retraction. Thus, in Text-fig. 5, the lower chart presents the curves of serum output of three specimens of blood from a rabbit. In order to vary the retraction rate each was placed at a different temperature to coagulate. Marked differences in rate are to be seen, the highest occurring in the specimen at the highest temperature; but the final readings, that is, the ultimate extents of retraction, in the three were the same. For comparison with these curves, the quantities of corpuscles which accompanied these serum yields at each reading during retraction, expressed in the same manner, are given in the upper chart of Text-fig. 5. Here again the curves demonstrate differences in rate, but the final values are not at all similar and vary also with the rate. It would appear that the total amount of corpuscles extruded from blood clot in retracting depends in some manner upon the rate of that process and in no wise upon its ultimate extent.

Corpuscle yield

At 37° C.

At 25° C.

At 17° C.

Serum yield

At 17° C.

At 25° C.

At 37° C.

Text-Fig. 5.

Further indication that the total corpuscle output from blood clot is an index of clot retraction rate is to be had in the following experiments, in which clot retraction rate is altered artificially by various means.

Table I contains the data of experiments utilizing the accelerating influence upon coagulation of contact of foreign substances (glass and copper) with the blood. The corpuscle yield was read upon specimens from three rabbits, the blood being contained during coagulation in paraffin-lined tubes and covered with

TABLE I.

The Accelerating Effect of Foreign Body Contact on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	per cent	*per cent*	*per cent*
Specimen 1 (tube paraffin-coated and with oil)...............	6	15	47
Specimen 2 (tube omitting paraffin coat).....................	17	27	
Specimen 3 (tube paraffin-coated and with oil, added piece of bare copper wire)...			74

TABLE II.

The Retarding Effect of Neutral Foreign Bodies on Retraction Rate.

	Corpuscle readings
	per cent
Specimen 1 (tube paraffin-coated and with oil)......................	47
Specimen 2 (the same as above; added one piece paraffin-coated copper wire)..	28
Specimen 3 (the same as above; added three pieces paraffin-coated copper wire)..	23

oil. With these as controls, readings of other specimens were made treated in all respects similarly, except that in two instances the paraffin lining was omitted, and in the third a fragment of copper was placed in the blood. The corpuscle yields in all of the latter were distinctly greater than in the controls.

The experiment in Table II made use of the retarding effect upon clot retraction of mechanical obstruction to the process. In comparison with a control reading, obtained as described in the above experiment, two other specimens from the same animal were tested in which there had been placed strands of copper

wire rendered neutral to coagulation by a coating of paraffin. The corpuscle reading from each of the latter was less than from the control and diminished in proportion to the number of wire strands present.

In Table III data are given concerning the retardation of clot retraction by exposure to air. Control readings were made as before in three rabbits and, for comparison, specimens were read from which the oil covering had been omitted. The corpuscle output was thereby lowered.

Agitation of the blood retards coagulation. In comparison with the usual controls in two rabbits (Table IV), specimens were tested similar to the others in all

TABLE III.

The Retarding Effect of Exposure to Air on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	per cent	*per cent*	*per cent*
Specimen 1 (tube paraffin-coated and with oil)................	65	59	24
Specimen 2 (tube omitting oil).............................	38	45	14

TABLE IV.

The Retarding Effect of Agitation on Retraction Rate.

	Corpuscle readings	
	Rabbits	
	A	B
	per cent	*per cent*
Specimen 1 (tube paraffin-lined and with oil)....................	47	31
Specimen 2 (the same as above).............................	49	
Specimen 3 (the same as above, stirred 1 min. after shedding).......	29	23
Specimen 4 (the same as above, stirred 2 min. after shedding).......		16

respects except that shortly after shedding the blood was slightly agitated. This had the effect in each instance of reducing the corpuscle yield.

Still further and surer proof of the significance of the corpuscle reading as an index of clot retraction rate is had from experience with the value in disease, for wherever retraction was found retarded as indicated by abnormally low intermediate serum readings, the total corpuscle yield was always depressed.

The means is presented, accordingly, of estimating the rate and the extent of blood clot retraction: The total fluid yield from clotted blood is separated into its serum-corpuscle fractions and, properly corrected, the corpuscle value indicates the rate and the serum value the extent of clot retraction.
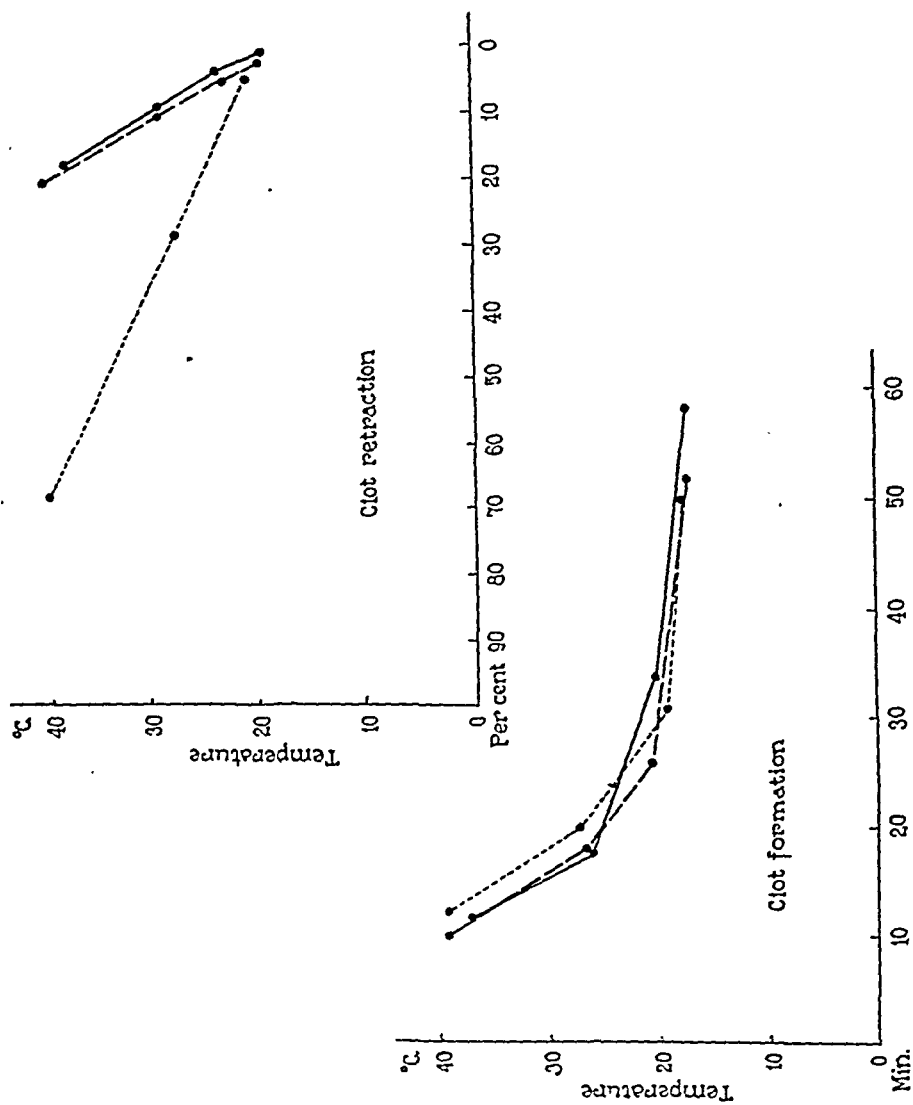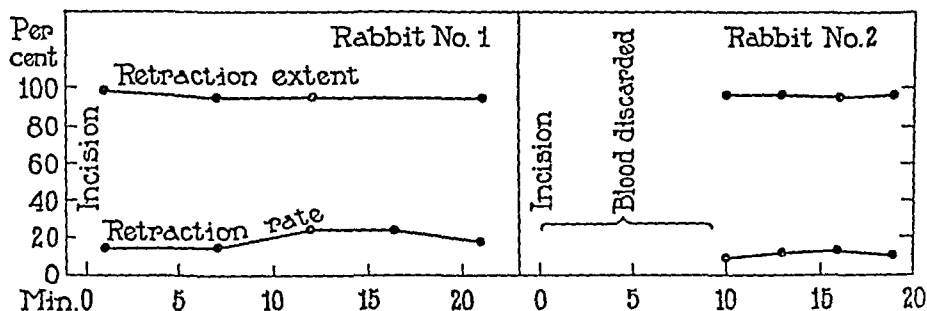
TEXT-FIG. 6.

Relation of Clot Retraction to Clot Formation.—We may proceed to examine the behavior of clot retraction in its relationship to that of clot formation.

In Text-fig. 6, on the right, are curves of retraction rate secured from blood of three rabbits. In each instance four specimens were taken and allowed to coagulate at temperatures extending from 17–38°C. It will be seen that not only does rate vary with temperature but the effect is proportional, *i.e.*, each curve is virtually a straight line. In Text-fig. 6, on the left, are curves of clot formation rate from these animals secured under the same conditions of temperature (see also Text-fig. 2, *A*). Here the relation between rate and temperature is represented by a line of hyperbolic form. Moreover, while the formation rates at corresponding levels are nearly alike in the three animals, one of them gave a retraction rate widely separated from those of the other two. Apparently, clot retraction and clot formation behave quite independently in rate.
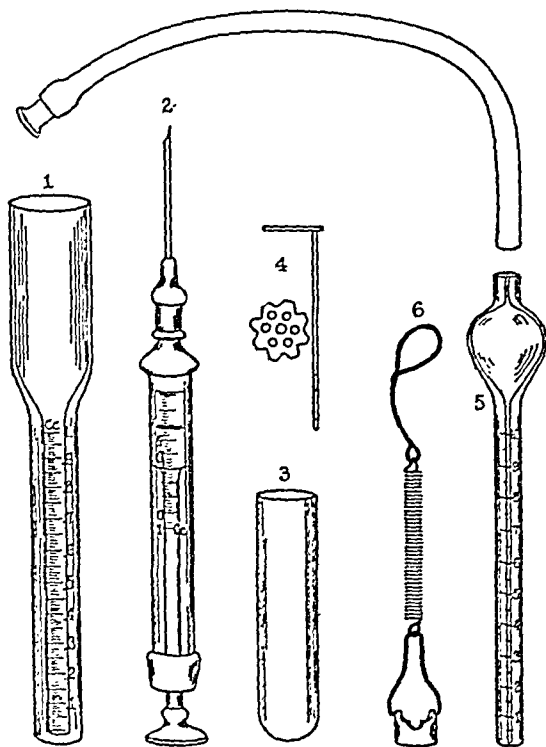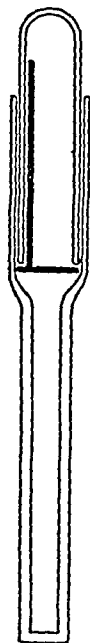

TEXT-FIG. 7.

This individuality of rate of the two phases of coagulation may be demonstrated in another way: Five specimens of blood from an animal were allowed to coagulate at different temperatures, thus, Nos. 1 and 2 at 17°C., No. 3 at 25°C. and Nos. 4 and 5 at 37°C. As coagulation proceeded, Specimens 2 and 4 were observed closely and at the completion of clot formation they were interchanged, *i.e.*, No. 2 was placed at 37°C. to undergo retraction, and No. 4 at 17°C. After coagulation was complete, the yields from all specimens were read (*Fig. 2*). The corpuscle yields from Nos. 1, 3 and 5 varied as would be expected with the temperature; the corpuscle yield of No. 2 was a duplicate of that of No. 5, and that of No. 4 was a duplicate of No. 1. Evidently, specimens which retracted at the same temperature retracted at similar rates, without reference to the temperature (rate) at which clot formation took place.

It has been demonstrated (Text-fig. 1) that clot formation is markedly accelerated by contact of the blood with the tissues. In Text-fig. 7 are given clot retraction readings in two rabbits, the specimens being taken successively from the same wounds. The curves are practically horizontal. From this it is evident that clot retraction is not affected as is clot formation by tissue kinase.

Apparatus.[1]—Record syringe (Text-fig. 8, No. 2), of 1.2 cc. capacity, graduated in 0.2 cc. divisions. Test-tube (Text-fig. 8, No. 3), of 1.0 cm. internal diameter and 5.0 cm. length. Graduated centrifuge tube (Text-fig. 8, No. 1), of 1.0 cc. capacity. Separating disc (Text-fig. 8, No. 4), which is a perforated metal disc of the same diameter as the test-tube. Hematocrit (Text-fig. 8, Nos. 5 and 6), described elsewhere (10, 11).

TEXT-FIG. 8.   TEXT-FIG. 9.

In preparation for the test the glassware is carefully cleaned, particular attention being paid the syringe that it contains no residue of blood and is dry. The test-tube is given a heavy lining of paraffin, thus: It is dipped into melted paraffin

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[1] These instruments may be obtained from the Gottlieb Greiner Company, New York City, with the exception of the hematocrit which is manufactured by the Arthur H. Thomas Company, Philadelphia, and Firma Arno Haak, Jena, Germany.

which has been cooled almost to the point of thickening, and on being removed is immediately placed inverted under cold running water. The external surface is then wiped free of paraffin and the inner surface examined to make sure that it has been coated entirely and is free from water.

Method.—The syringe is filled to 1.2 cc. with blood (for the rabbit see technique of collecting blood above) and 1.0 cc. of this is deposited precisely in the bottom of the test-tube (thus is avoided error from the dead space air bubble in the syringe). A few drops of mineral oil are then added to cover the specimen and it is placed at 37°C. for coagulation.

An hematocrit test is now performed.

From 5 to 6 hours after collection of the specimen, the test-tube is removed from the incubator, covered with the separating disc and inverted into the centrifuge tube (Text-fig. 9). This is then centrifugalized for 30 seconds at the lowest speed (about 500 R.P.M.) necessary to displace the fluid contents of the test-tube completely through the separating disc into the graduated tube. Now, the test-tube, separating disc and blood clot are removed, and the graduated tube, containing the fluid yielded from the clot, is centrifugalized at high speed (about 3000 R.P.M.) for 5 minutes to separate corpuscles from serum.

The amounts of corpuscles and serum are read from the scale on the centrifuge tube (Fig. 2), and these values are corrected with reference to the hematocrit result, to give the final readings, as follows:

If x = final serum reading, representing retraction extent,

y = final corpuscle reading, representing retraction rate,

a = serum reading from tube; for example, 0.56 cc.,

b = corpuscle reading from tube; for example, 0.18 cc.,

and c = amount of corpuscles in whole blood; for example, where the hematocrit reading is 40 per cent, the corpuscle content of the specimen is 0.40 cc.,

then $x = \dfrac{a}{1.00 - c}$, for example $x = \dfrac{0.56}{1.00-0.40} = 0.93$ (read 93 per cent),

and $y = \dfrac{b}{c}$ for example $y = \dfrac{0.18}{0.40} = 0.45$ (read 45 per cent).

Certain technical points require emphasis:

It is of first importance in securing the specimen that no more than 20 seconds shall elapse between its leaving the circulation and entering the test-tube, for agitation of the blood in the early stages of coagulation produces marked reduction in coagulation rate and renders the reading misleading (Table IV). This small quantity of blood may be obtained rapidly enough when withdrawn by venepuncture, but in the rabbit where it is collected from the pool of blood issuing from an incision the task is often difficult. When there is any question about the rapidity of collection, a second specimen should be taken and, if the reading is higher, adopted. Once the blood is in the tube further haste is not imperative, although no more than 2 minutes should intervene before placing it in the incubator.

If hemolysis occurs and the serum is found tinted, no reading is to be made, since bacterial activity has probably altered the specimen. For this reason the incubation is limited to 6 hours.

Where it is obvious from inspection after incubation that clot retraction has occurred to a slight extent only, it is better to remove the accumulated serum by pipette for measurement rather than by centrifugalization, for such clots are soft
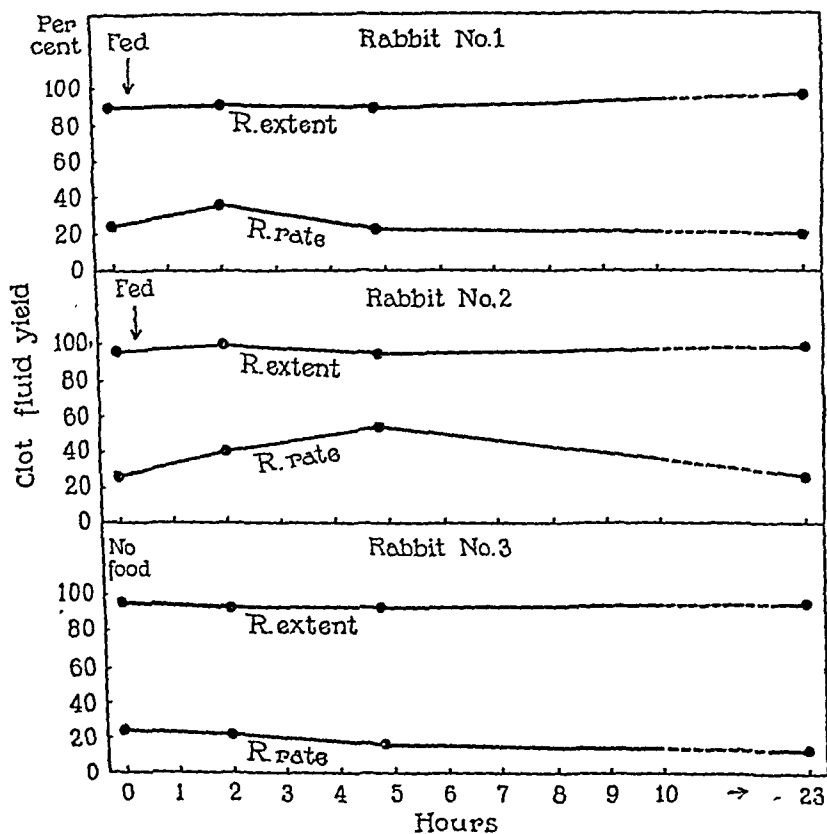
TEXT-FIG. 10.

and give up additional fluid readily on pressure. Where clot formation fails or takes place only in portions of the specimen, it is of course impossible to measure clot retraction and fluid readings become valueless. In these cases there will usually be found upon the separating disc either no clot at all or misshapen clot fragments; even where the clot appears normal the condition may be detected,

since the serum reading is abnormally low (under 87 per cent) and the corpuscle value high (over 30 per cent). Such a result is, of course, indicative of an exceedingly defective coagulation and is of value qualitatively.

At the time of examination the subject should be fasting. The apparent accelerating effect of food ingestion upon clot retraction is illustrated in Text-fig. 10.

Results.—Records are at hand of 810 readings of clot retractility. The normal rate of clot retraction lies between the values 40 and 60 per cent (corpuscle output) and the normal extent between 87 and 100 per cent (serum output). In disease, instances were found of all grades of depression of these values, to 0 per cent in non-retractile blood clot.

The margin of error from various technical sources allowed in the interpretation of readings is 10 per cent of the rate value and 2 per cent of the extent value.

DISCUSSION.

The factor here measured as clot formation rate is but another expression of the same function of the blood coagulative process as the clotting rate values of other methods. This is shown by their similarity of behavior in the presence of various disturbing influences, such as tissue kinase and foreign body contact with the blood, but more particularly by the type of their reaction to temperature change. Thus, the temperature curve of clot formation as given in Text-fig. 2, at *A*, runs parallel with those at *B*, *C* and *D*, which are constructed from data published respectively by Lee and White (7) with the tube inversion method of Howell, by Addis (6) with his modification of the hanging drop method of Brodie and Russell, and by Dale and Laidlaw (12) with their shot tube procedure. However, judging from the positions occupied by the curves relative to each other, the end-point of clot formation is situated further along in the process of coagulation and would logically have the advantage of magnifying changes occurring in coagulation rate. In this procedure, also, the conditions under which the specimen resides while coagulating are nearly neutral, and the rate obtained may be considered as practically basic for the blood examined.

Sedimentation of corpuscles in the drop of blood tested is a phenomenon especially to be noted. It has been observed in specimens of coagulating blood by others, and is the cause of production of the

"buffy-coated" blood clot (13, 14). Addis (15) found it associated with prolonged clotting time in extremely ill, septic patients. Lee and White (7) noticed it in various diseases, and so frequently was it accompanied by delayed clotting time, that the phenomenon was used as confirmatory evidence of the latter condition. Sedimentation has been found here also followed by an extended end-point of clot formation, but it probably has particularly to do with the beginning-point and indicates delay in the onset of coagulation. This can be shown as follows: If a specimen of blood is chilled directly after collection, the corpuscles will soon commence to gravitate and this will continue until the temperature is elevated and coagulation is allowed to proceed; should, however, the chilling be postponed for 1 minute after shedding, to allow coagulation to begin, and is then instituted, sedimentation will not take place much as the clotting end-point may be delayed by the low temperature. Also, it has been found that, where corpuscle sedimentation occurs in disease, examination of the blood by means of the wire loop of Buckmaster (5) demonstrates marked delay in the appearance of certain alterations in the specimen peculiar to the onset of coagulation. In the gravitation of the corpuscles, accordingly, is presented a means of detecting retarded initiation of coagulation, a factor in addition to the estimation of clot formation rate.

Clot retraction is a function which depends upon physical principles quite separate and distinct from those underlying clot formation, since, as has been demonstrated, they behave differently in the presence of various disturbing influences.

It has been necessary to resort to indirect means for the estimation of clot retractility. Direct measurement of size alterations in the clot itself with any degree of accuracy is well nigh impossible, since the pattern of retraction is extremely variable, particularly in the early stages, and measurement in one or two diameters alone is not sufficient. However, the amount of fluid yielded from the clot during retraction is as logical a measure and more readily applied; either procedure must take into consideration the plasma-corpuscle ratio of the blood and the amount of corpuscles extruded with the serum, in order to narrow the range of normal values and distinguish the abnormal. The reason is not apparent for the parallelism found between the rate of clot retraction and the quantity of corpuscles which escapes from

the clot. This has probably something to do with the mechanics of fibrin retraction. In early tests, an additional serum reading was made 1 hour after collecting the blood and compared with the final serum reading as an indicator of clot retraction rate. But this was abandoned when the significance of the corpuscle output was appreciated, and, not only has the technique been simplified thereby, but the corpuscle reading has proven much more delicate in determining changes in rate.

SUMMARY.

The gross phenomena of normal blood coagulation have been studied for the purpose of obtaining methods for estimating certain characteristics of coagulation in connection with a malignant tumor of the rabbit. The technique is described for the estimation of clot formation rate and of clot retraction rate and extent. The range of normal and abnormal values secured by these procedures is given.

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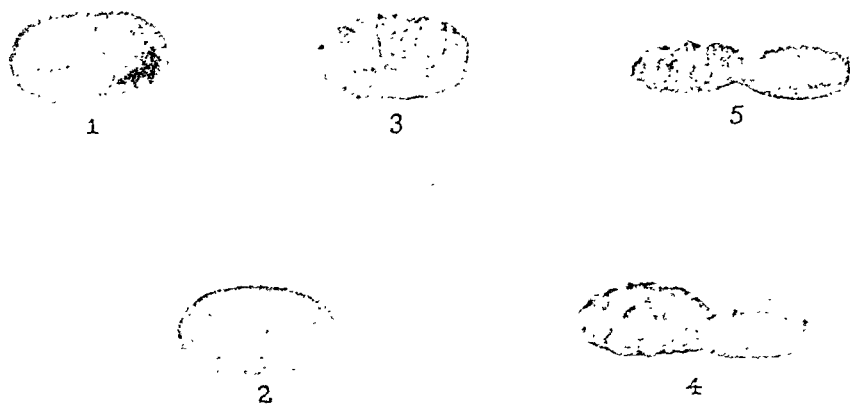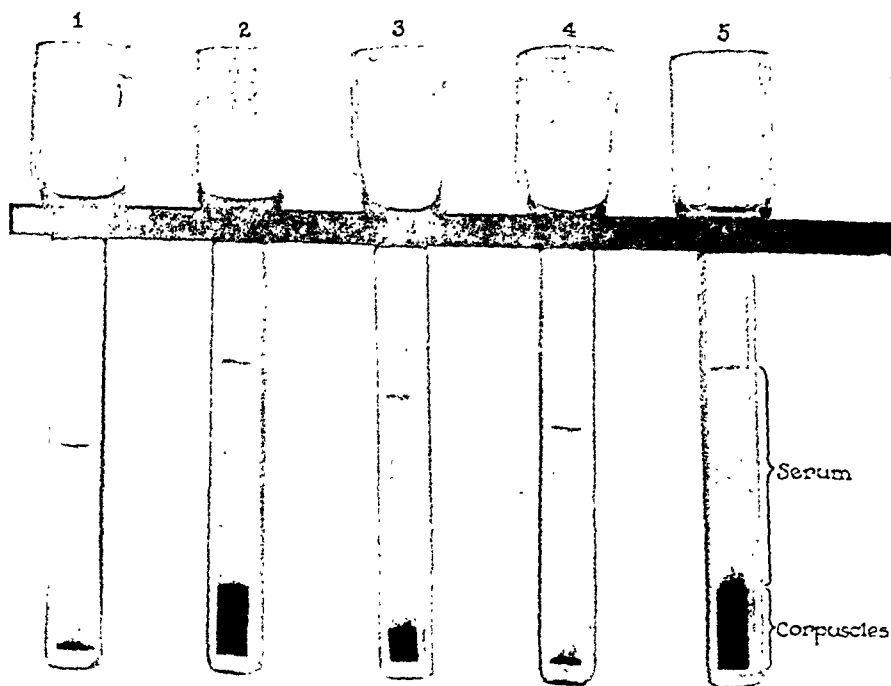15. Addis, T., *Edinburgh Med. J.*, 1910, v, 38.

Fig. 1.

Fig. 2.

STUDIES IN BLOOD COAGULATION.

II. Blood Coagulability in Malignant Tumor and Other Diseases of the Rabbit.

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Alterations in the coagulability of the blood will be described which occur in various stages of the growth and extension of a malignant tumor of the rabbit, and, as a basis of interpretation, there is explained also the behavior of coagulation in other diseases and abnormal states of this animal. The methods of test employed have been described (1), and include estimation of clot initiation and clot formation rate and the rate and extent of clot retraction.

I. Disease in General.

Relation of Blood Coagulability to Presence and Extent of Disease Lesions.

Experimental.—100 adult male rabbits, taken from stock as received from the breeders, were placed under observation for 1 or more weeks.[1] All grades of physical condition were presented, although the great majority were free from external evidences of disease. The blood coagulability of each animal was then tested, and autopsy examination was made within 24 hours afterward. A comparison of the readings with the anatomical findings follows.

Results.—As regards clot formation rate, the readings in almost every instance were within the limits of normal variation, *i.e.*, between 4 and 8 minutes. Five exceptions to this presented, and all of these were in extreme stages of disease: Two gave clot formation time of 10 minutes, and showed at autopsy fatty degeneration of the kidneys and profound cachexia, which changes were probably secondary to an occult disease process. One rabbit with extensive coccidiosis of the

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[1] These rabbits are part of a larger group being used for other experiments in the study of normal and abnormal organ weight variations.

liver, another with snuffles complicated by septicemia and a third markedly emaciated but without discernible lesions gave delayed clot initiation and an irregular clot formation allowing of no distinct reading.

The extent of clot retraction was also normal in the majority of the animals, *i.e.*, reading between 87 and 100 per cent. In the last

TABLE I.

Autopsy finding		Analysis of groups on a basis of clot retraction rate				
		40–60	30–40	20–30	0–20	No reading obtainable
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Rabbits grouped on a basis of stress of disease	11, without lesions and in good physical condition...................	91	9			
	10, with healed lesions of various types.........................	80	20			
	24, with coccidial lesions, small and of questionable activity.........	25	33	29	13	
	13, in process of recovery from various types of infections..........		32	60	8	
	18, with purulent infections, snuffles, mastoiditis, etc.................		5½	11	78	5½
	8, with coccidial lesions, numerous and obviously active............			40	50	10
	4, emaciated, with fatty degenerative lesions of kidneys..........				25	75
	2, emaciated, with active parasitic lesions of kidneys...............				100	
	2, emaciated, with focal lesions of appendix......................				100	
	3, emaciated, but without discoverable disease process.............				67	33

three rabbits above mentioned occurred the only abnormal values, 85, 85½ and 81 per cent, respectively.

Clot retraction rate, on the other hand, was extremely variable, both in rabbits obviously ill and those without symptoms. The autopsy findings are correlated statistically in Table I with the retraction rate values. On the left, the rabbits are grouped according

to the type of disease found and the groups are arranged in order on a basis of degree of physical deterioration (disease stress) evident. On the right, analysis is made of each group from a standpoint of the clot retraction rates, and for this purpose four divisions are used. It will be seen that in the highest rate division, 40 to 60, fall 91 per cent of animals without lesions and in good physical condition, also, 80 per cent of those with healed lesions only and 25 per cent with insignificant coccidial lesions. As analysis proceeds thus from group to group dealing with disease of increasing severity, the distribution of rate values plainly shifts across the scale, and the last five groups lie entirely within the lowest rate division, 0 to 20. The animals which fall principally in the intermediate divisions, 20 to 30 to 40, are those with moderately active disease or in process of recovery from severe infections. Retraction rate could not be measured in three rabbits, those last referred to above, because of the fragmentary nature of the coagulative process.

Five rabbits are excluded from the table for special mention: Two had been inoculated intratesticularly with the spirochete of yaws and had an early, active orchitis. At autopsy no other lesions were found. The retraction rates were normal, 42 and 48 per cent. Two rabbits had well developed goiter, were otherwise in good condition and gave readings, 45 and 56 per cent, within the normal range. One rabbit although a female was included in the series to illustrate a point of interest. She was abundantly fat and sleek and presented nothing pathological beyond a small, healing *cuniculi* papule on the genitalia. Nevertheless, the clot had an extremely low retraction rate, 9 per cent; at autopsy pregnancy at about the 10th day was discovered.

### *Relation of Blood Coagulability to Course of Disease.*

Experimental.—The behavior of blood coagulation was investigated during the course of various abnormal and diseased states, including instances of spontaneous infections, non-bacterial lesions and artificially induced affections of the blood coagulative mechanism. In each case, periodic tests were made and symptoms recorded, with autopsy examination at the termination of the experiment.

Findings.—Brief protocols of typical examples of each disease are given:

Snuffles.—Text-fig. 1 is the chart of a rabbit which had been followed for some time because of chronic snuffles. The infection persisted for about 2 months, gradually cleared, and the nasal discharge had ceased, when abruptly a relapse set in with profuse discharge and sneezing and the animal passed through a
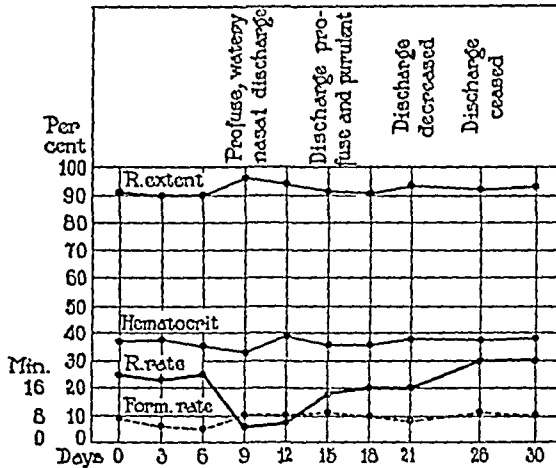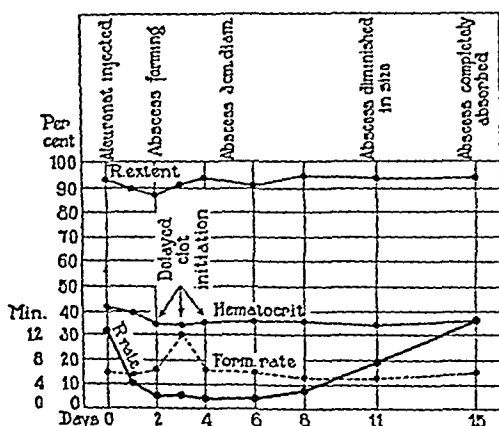

TEXT-FIG. 1.
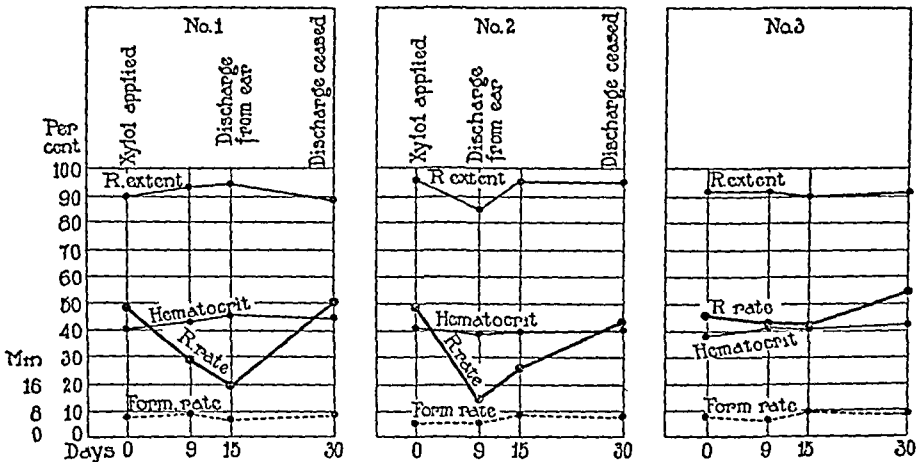
TEXT-FIG. 2.

second typical course of the disease, this time lasting 3 weeks. At autopsy, the nasal passages were free from inflammation; the right middle ear spaces contained a small quantity of inspissated pus. There was no other disease. Evidently the rabbit had had chronic otitis in addition to the attacks of snuffles. The period of snuffles remission only is included in the chart of blood findings. The rate of

retraction had been distinctly below normal and dropped still farther with the redevelopment of rhinitis. Later, as the infection cleared, the curve rose to its previous level. The readings of retraction extent and clot formation rate showed throughout no variations beyond the normal range.

Auditory Canal Abscess.—The charts are given in Text-fig. 2 of three rabbits from a group of fifteen which were being followed as controls for other purposes. At this time xylol was being used routinely painted on the inner surface of the ear to produce vessel dilatation for the collection of blood specimens, and in two instances, Rabbits 1 and 2, the xylol extended accidentally into the auditory meatus. The applications were discontinued immediately in favor of other methods, but the inflammation in the canal persisted and an abscess developed in its recesses. Rupture occurred in 2 weeks, with drainage of pus for another week. Rabbit 3 is given for comparison, for here no irritation arose, aside from a
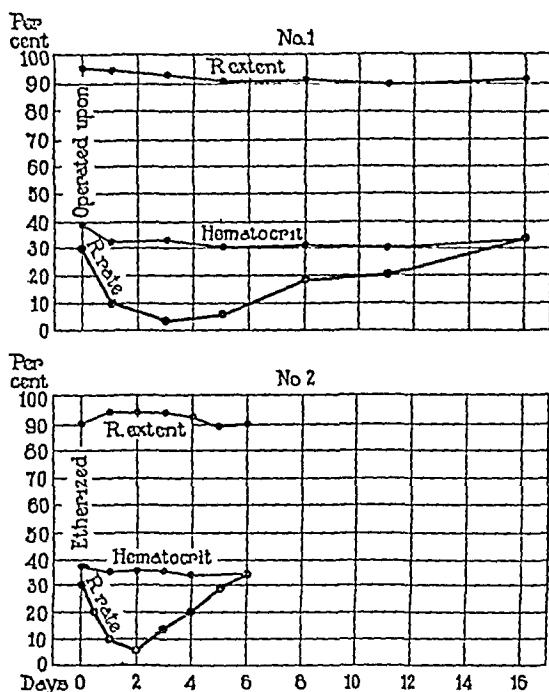

TEXT-FIG. 3.

slight erythema of the pinna. From the charts, it is to be seen that in the cases of the first two rabbits clot retraction rate fell after xylol application to a low level on the 15th and 9th days respectively, and with rupture of the abscess the reading began to rise and reached normal with the healing of the lesion. Retraction extent in the first rabbit was elevated in the early stage of inflammation, but in the second, where the rate drop had been more sudden and pronounced, the extent reading also fell temporarily. Both rate and extent of clot retraction in the third rabbit remained during this period within the normal limits. Clot formation showed no abnormalities in any of the animals.

Subcutaneous (Sterile) Abscess.—Six rabbits were injected either subcutaneously or intrapleurally with 0.5 cc. of a 10 per cent suspension of aleuronat. In each, pus accumulated at the site of injection, and in two of the animals which were allowed to live for 3 weeks the abscess was absorbed and no trace remained except

slight local increased vascularity.   The record of one of the latter is represented in
Text-fig. 3, the two being similar in all respects.   The curve of retraction rate fell
sharply within 24 hours after injection and remained at a low level for 6 days during
the period of abscess development.   Then the curve began to rise and by the 15th
day was at normal, when the lesion was healed.   The extent of retraction did not
leave the normal range.   Clot initiation and formation were delayed slightly at
the height of the inflammatory reaction.

Operative Incision.—Four rabbits were operated upon under ether anesthesia
with sterile precautions for the purposes of other work.   In two the spleen was
removed and in the others laparotomy only was done, as control.   The wounds

TEXT-FIG. 4.

healed *per primam*.   The coagulation curves of one of the control rabbits, which
were typical of all, are given in Text-fig. 4, No. 1.   Immediately after the operation
the rate of retraction dropped and reached a low point on the 3rd day, when it
began to return and attained its original level on the 16th day.   The extent of
retraction remained stationary throughout.   No clot formation readings were
made.

Ether Anesthesia.—In order to ascertain the part played by ether in the preced-
ing results, two animals were anesthetized for a period of ½ hour.   The subsequent
behavior of clot retraction in both was the same; the curves of one are given in

Text-fig. 4, No. 2. The rate became retarded in a manner similar to that in the chart above, but returned much more rapidly to normal.

Nephritis.—The chart is given in Text-fig. 5 of a normal rabbit which developed nephritis spontaneously while under observation. The animal had been in good health and gained steadily in weight until the 4th week of observation, when it began to grow thinner without other symptoms. 1000 gm. were lost before death 3 weeks later. At autopsy, the body tissues were found dehydrated and wasted; the only lesion presenting was with reference to the kidneys. These organs were markedly swollen, pitted and intensely yellow, with a fine surface stippling. On section, the cortices were found widened, of an opaque yellow color and without architectural markings. The medullæ were pale and presented radial grey streaks. From the chart, it will be seen that clot retraction rate
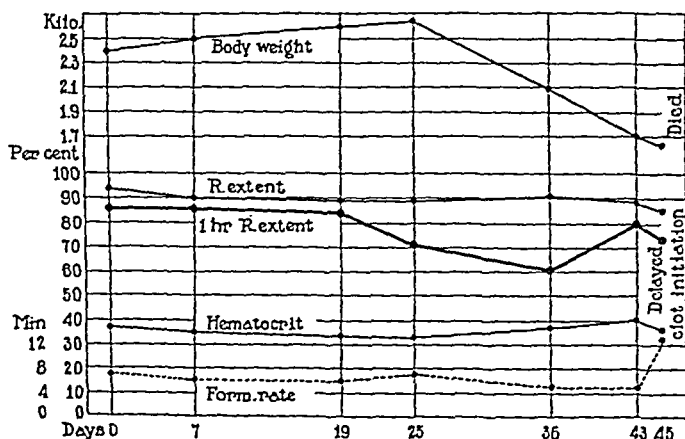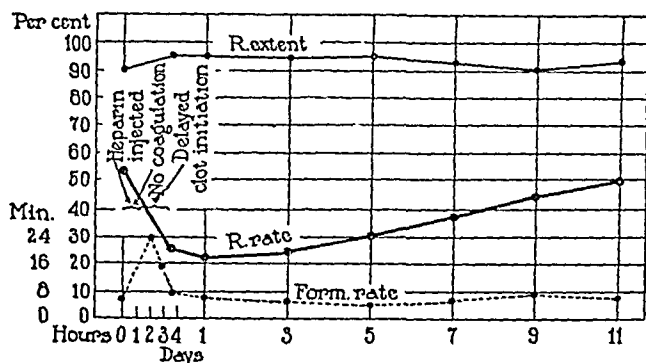

TEXT-FIG. 5.

(estimated by a 1 hour serum reading in comparison with the final serum output, a less sensitive index) showed marked retardation on the 25th and 36th days, a return toward normal on the 43rd day, and another drop before death. Retraction extent and formation rate showed no abnormalities until the final reading, when the latter was delayed.

Specific Affections of the Blood-Coagulative Mechanism.—Effort was made to reproduce experimentally conditions of blood coagulability imitating those in the hemorrhagic diatheses, hemophilia and thrombocytopenic purpura.

The properties of the blood in hemophilia are said to be obtained temporarily in animals by the injection of heparin (2), a phosphatide extracted from the liver by the process of Howell, that is, clotting time is prolonged but, when coagulation ensues, the clot retraction appears normal. In the present study seventeen rabbits

were used and heparin,[2] at a concentration of 0.5 per cent in physiological saline solution, was injected intravenously in amounts from 5 to 40 mg. per kilo body weight. Both single and series of doses extending over long periods were employed. In none of the animals appeared symptoms of any sort referable to the drug, nor did microscopic examination of the bone marrow, spleen, liver and other organs reveal changes. Marked alterations in blood coagulability were presented however, shown typically in Text-fig. 6. Here a single dose of 17 mg. per kilo was injected. The blood, examined immediately and again after 1 hour, failed to coagulate. 2 hours after treatment a specimen gave moderately delayed initiation of coagulation and formation time of 23 minutes. At 2 hours, 40 minutes, these two functions were still tardy but became normal 1 hour later. Although clot retraction was observed to occur in portions of specimens as early as 2 hours after injection, the process was so imperfect as to exclude estimation. At 3 hours, 40 minutes, the first measurement was obtainable and gave a considerably reduced
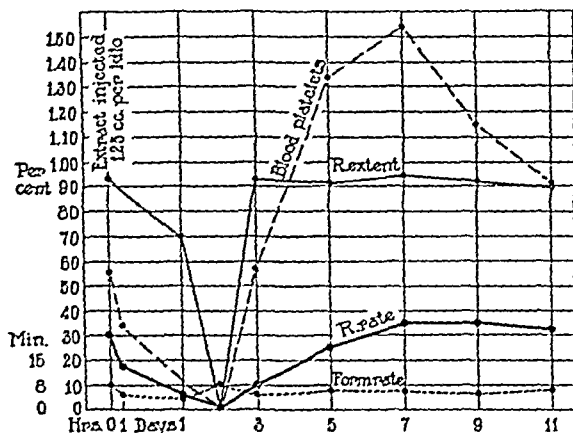

TEXT-FIG. 6.

rate and a normal extent. Further depression in rate was found after 24 hours, and then this function returned gradually to its original level by the 11th day. The other factors of coagulation were normal after the initial interference. These alterations in the blood varied in degree among the animals with the dose employed. After repeated treatments (20 in 60 days, of 40 mg. per kilo) rabbits appeared to have become somewhat resistant to the drug, although this was not consistent.

The pneumococcus extract of Avery and Neill (3) is known to have a specific lytic effect upon the blood platelets and to produce in mice a transitory purpuric condition. I am indebted to Dr. Avery and Dr. Julianelle of The Rockefeller Institute Hospital for a supply of the extract. This was first warmed to remove the hemolysin, and amounts from 0.25 to 3.50 cc. per kilo body weight were

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[2] Heparin may be obtained from Hynson, Westcott and Dunning, of 150 Nassau Street, New York City, and Baltimore, Md.

injected intravenously in twelve rabbits.   No symptoms were observed, although one rabbit receiving the highest dose was found dead after 18 hours.   No purpura or tendency to bleed (as indicated by the incisions in the ear) was evident, even

TEXT-FIG. 7.
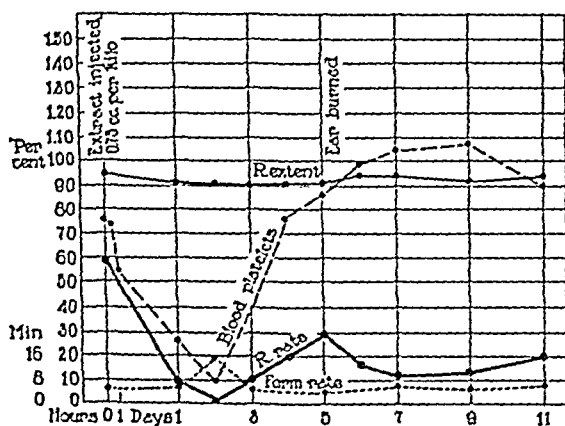
TEXT-FIG. 8.

when the platelets were practically absent from the blood stream.   Microscopic study of the bone marrow, spleen, liver, lymph nodes and other organs and tissues discovered no changes.   Charts of two of the rabbits are given (Text-figs. 7 and 8). Curves have been added representing the platelet content of the blood; these values

were obtained with the thrombocytocrit,[3] an instrument recently described (4), and give estimations of platelets volumetrically instead of numerically as customary. It will be seen that platelet lysis commenced immediately after injection of the extract and reached an extreme degree on the 2nd day. The platelets then reaccumulated rapidly and in great excess, attaining a maximum between the 7th and 9th days, after which their volume returned to normal. This behavior is similar to that described in the mouse by Julianelle and Reimann (5). In Text-fig. 7, following the injection of 1.25 cc. extract per kilo, the rate and extent of clot retraction began to fall within 1 hour, and continued to descend until the 2nd day, when the clot, although of firm consistency, failed to retract. The next day retraction had returned to normal in extent but took place at a very slow rate. The rate returned to its original level by the 7th day. Clot formation showed no departure from normal throughout. In Text-fig. 8, following the injection of a smaller dose, the retraction rate fell as before but its extent remained unaffected. By the 5th day the rate had begun to ascend, but once more was depressed coincidently with the occurrence of a slight accidental burn of the rabbit's ear while warming it to secure blood, and the typical reaction to the extract was thus altered. Clot formation became slightly retarded on the 2nd day. These changes in blood coagulation were found to vary in degree among the rabbits approximately with the size of dose employed. Even with as small an amount as 0.25 cc. per kilo retraction rate was markedly reduced, but the extent of retraction was affected only with the larger amounts of the extract. A close parallelism was demonstrated between retraction rate and the platelet content of the blood, even to the extent that thrombocytosis was associated with a slightly hypernormal retraction rate. The relation held, however, only in animals free from infections, for, as in Text-fig. 8, depression in rate took place under conditions of disease without regard for the platelet reading. The extent of retraction was interfered with only when the platelets reached an exceedingly low level.

II. Tumor.

The tumor which forms the basis of these experiments is an epithelioma of variable malignancy (6, 7). It arose spontaneously in a rabbit and has been carried in this species for purposes of study by successive transplantation. Testicular inoculation of tumor tissue, as routine, gives rise invariably to local neoplastic growth. Metastases develop in a high proportion of instances, often very profuse and resulting fatally within a period of from 20 to 80 days. In others, after a certain amount of growth, the neoplasm becomes necrotic and is absorbed, with survival of the animal.

[3] Obtainable from Arthur H. Thomas Co., Philadelphia, and from Firma Arno Haak, Jena, Germany.

Examination of the blood coagulability has been carried out in 58 rabbits inoculated with tumor, tests being made at regular intervals throughout the course of the disease.   In many cases the results have been difficult to interpret because of complication of the picture by the effects of spontaneous infections, most frequently appearing where the tumor ran a long and severe course and resulted in physical depletion of the animal.   However, uncomplicated cases are abundantly at hand, and typical examples will be described.
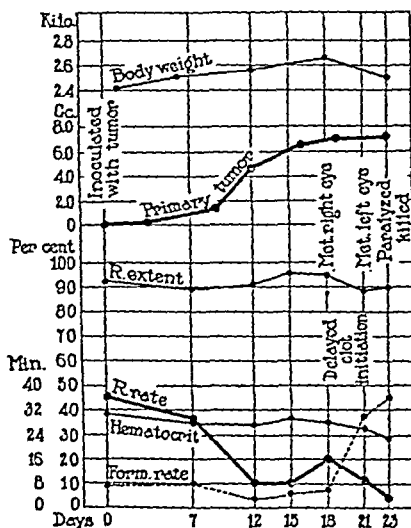
TEXT-FIG. 9.

A very malignant form of the tumor is illustrated by an animal whose chart is given in Text-fig. 9.   The primary tumor grew rapidly and on the 18th day a metastasis was discerned in the right iris.   This developed vigorously and 3 days later the left eye was similarly involved.   During this period the rabbit lost in strength and ate little.   The hind quarters were found paralyzed on the 24th day and the experiment was terminated.   At postmortem examination the general state of nourishment was found reduced but not depleted.   The primary tumor measured 1.5 by 1.5 by 5.0 cm., had completely destroyed the testicle and invaded the spermatic cord.   Extensive involvement by metastases of the abdominal viscera was presented, also, of both eyes, lymph nodes of the neck, femoral bone marrow on both sides and spinal column.   The blood readings obtained are shown in the chart, a curve being added to represent the rate of growth of the primary

tumor as estimated by measurements during life (8). Shortly after inoculation, at a time when the initial tumor could be felt in the testicle as a pea-sized nodule, a distinct depression was present in clot retraction rate, and this value continued to fall still farther. On the 18th day the reading tended to return somewhat toward normal but soon fell again and reached a very low level at the end of the experiment. The curve of retraction rate, thus, is seen to represent practically an inversion of the growth curve of the tumor. Retraction extent remained within the normal range throughout. Clot initiation and formation were normal until the last 2 days when marked delay occurred in these functions.
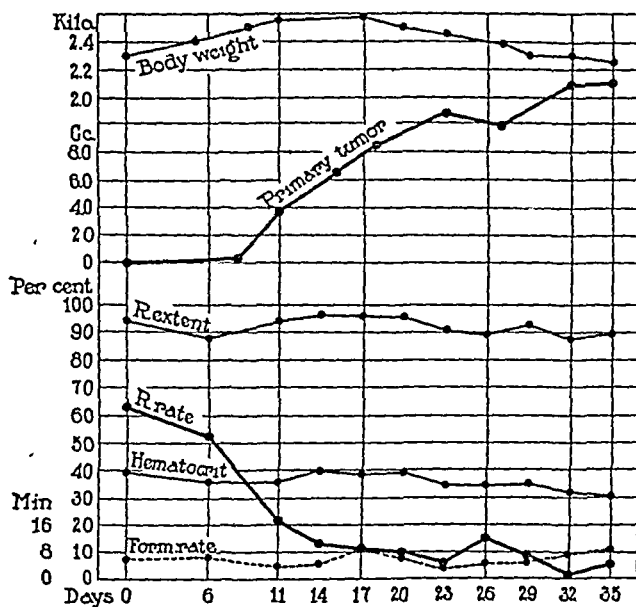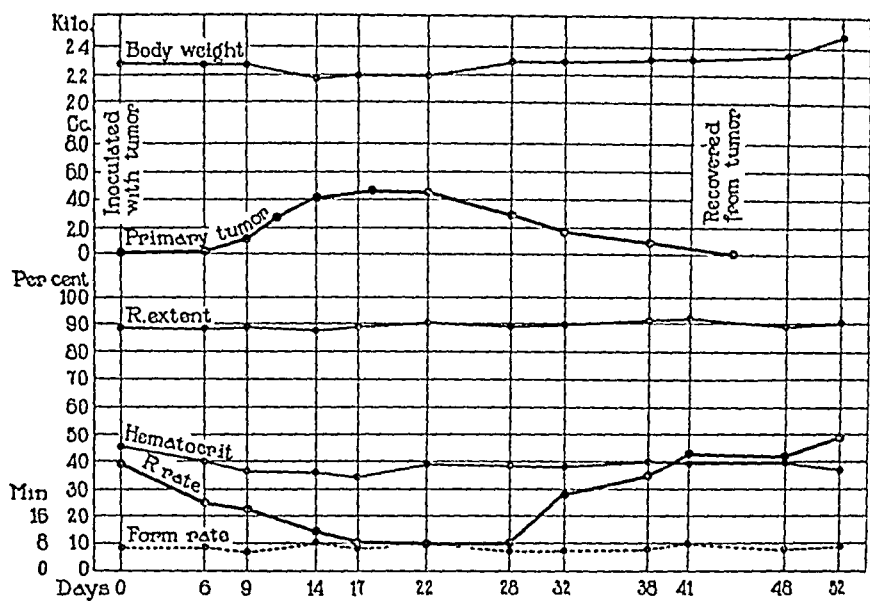

TEXT-FIG. 10.

An instance of more gradually extending but severe neoplastic disease is given in Text-fig. 10. The primary tumor developed steadily to large size. On the 19th day the first metastasis was discovered and others followed. From this time on the animal lost in weight and by the 35th day had become emaciated and weak, when it was killed and autopsied. The body tissues were wasted. The primary tumor completely distended the scrotal sac, and tumor nodules were found in the spermatic cord and retroperitoneal lymphatics. The adrenals were largely replaced by metastases; the omentum contained clusters of them. Nodules were present also in the mediastinum, posterior lymphatics of the neck and right iris, and the nasal cavities and sinuses were filled with neoplastic growth. A nodule had entirely destroyed the hypophysis, eroding the sella turcica. In the chart it will be seen that clot retraction rate began to diminish at an early stage of tumor

development and dropped progressively with its further extension, to a low value at death. Here again, the curve of this function parallels inversely the tumor curve. Fluctuations which occurred in the extent of retraction and in clot formation rate were minor and lay within the limits of normal.

A comparatively benign course of the disease is illustrated in the cases of Text-figs. 11 and 12. The primary tumors grew to moderate (Text-fig. 11) or large (Text-fig. 12) size, and then receded without giving rise to discoverable metastases. The charts show a progressive depression of clot retraction rate during the early stages of the disease, and a return to normal as the tumors become absorbed. Retraction extent and formation rate showed no definite changes.

The rabbits whose charts are given in Text-figs. 13 and 14 are presented as types with complications. In the first, the testicular tumor developed to moderate proportions and metastases were felt in the abdominal wall on the 19th day. The iris of both eyes became involved. After the 25th day the tumors ceased to grow and were eventually absorbed. 48 days after inoculation an attack of snuffles set in with sneezing and profuse nasal discharge. A few days later the experiment was terminated. At postmortem examination the nasal cavities were found filled with pus; other than this and the scar of the healed primary tumor no lesions were present. The chart (Text-fig. 13) indicates that during the period of tumor activity retraction rate was depressed, and showed a secondary depression while the necrotic tumor tissue was being absorbed. After returning finally to a normal level, it dropped a third time at the onset of upper respiratory infection. Other factors in the blood tests were normal throughout.

The rabbit of Text-fig. 14 pursued an extended and rather severe form of the disease. The testicle containing the primary tumor was withdrawn into the abdomen soon after inoculation and the growth of the tumor could not be followed. On the 28th day appeared a metastasis in the right iris and others followed in great numbers,—with masses of tumor to be felt in the deep tissues of the neck and in the abdomen. Growths developed at the gingival margins of the upper molar teeth, protruding into the mouth to such an extent as to interfere with eating. From the 30th day on the rabbit became thinner and ultimately cachectic, although it remained vigorous at all times. The experiment was terminated 58 days after inoculation. Autopsy: The tissues were everywhere wasted. An enormous primary tumor lay within the peritoneal cavity, and massive collections of metastases involved the retroperitoneal lymphatics, adrenals, kidneys, spleen, lungs, mediastinum, thymus, neck muscles and lymphatics, hypophysis, both eyes and the maxillary antrum, with infiltration of the alveolar processes and gums. All tumors, however, were thickly encapsulated and entirely necrotic. Evidently the neoplastic process had been arrested for some time, and the cachexia was the combined result of absorption of necrotic tumor tissue and the inability of the animal to take food properly. In the chart, retraction rate is indicated by a 1 hour serum reading in comparison with the final, and this value is seen to have fallen slightly during the period of metastasis (30th to 43rd days), and to have risen to a
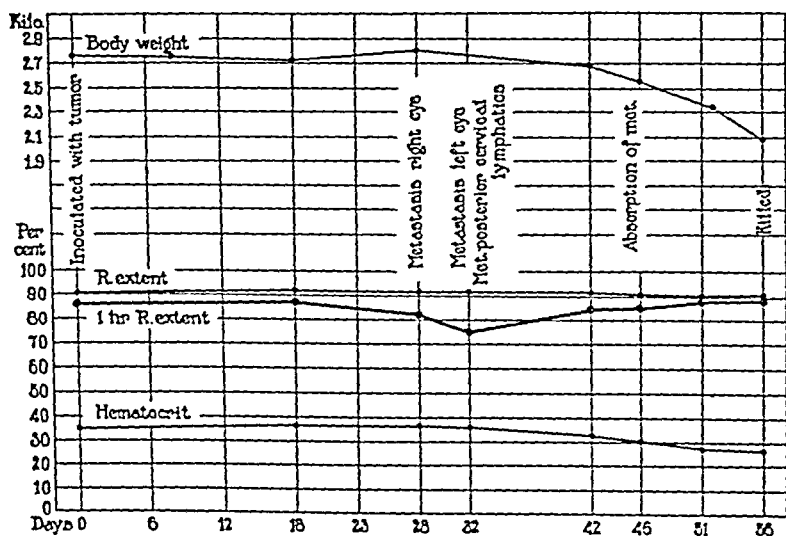
TEXT-FIG. 11.
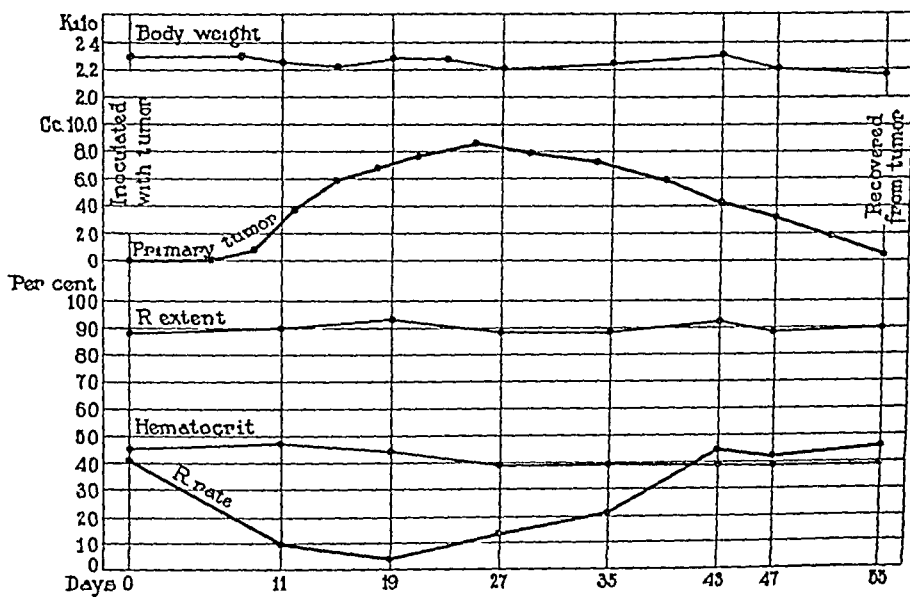
TEXT-FIG. 12.
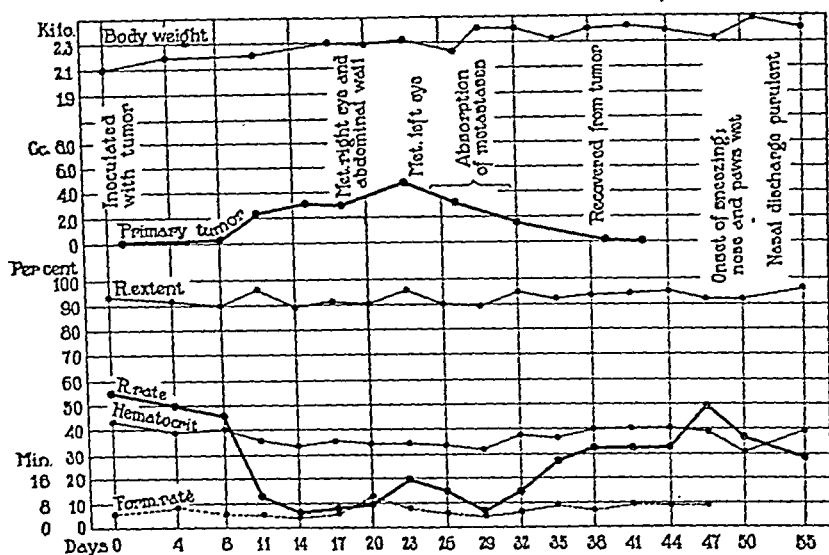
TEXT-FIG. 13.

TEXT-FIG. 14.

high position the last week of the experiment.   Other factors in the tests showed no alterations of note.

<center>DISCUSSION.</center>

The reports in the literature are very inconsistent with regard to blood coagulability in disease in general, excluding the hemorrhagic diatheses, but where authors have used carefully controlled methods of testing the results indicate that coagulation rate and the retraction of the clot as observed qualitatively remain quite normal.   Addis (9), in examining 112 patients, all of whom were seriously ill and presenting various sorts of disease both bacterial and non-bacterial in nature, found changes in coagulation rate in 30 per cent, and these were in an extremely critical condition when the blood was tested.   Lee and White (10) conducting a similar investigation also obtained alterations only in the critically ill, and then not constantly.   Pneumonia is an exception, for Dochez (11) and others have demonstrated consistent delay in clotting rate during its progressive stages; this is probably to be accounted for in the presence of the pneumococcus toxin (Avery) and its specific action upon elements of the blood connected with coagulation.   Imperfectly retracting or non-retractile blood clot has been reported to occur occasionally in certain infections, i.e., diphtheria, pneumonia, miliary tuberculosis, nephritis, ulcerative colitis, smallpox, etc., and these, too, have been cases of unusual severity. Thrombocytopenic purpura hæmorrhagica is the only condition which presents non-retractile blood clot with any regularity.   The results of the present work in diseases of the rabbit agree with these views, i.e., alterations in the rate of clot formation and extent of clot retraction were found confined to rapidly progressing pathological conditions, at the height of the disease and at terminal stages, and here inconstantly.   They failed entirely to appear in gradually developing maladies, even where profound emaciation and weakness were reached.

One factor of the blood coagulative process, however, the rate with which the clot retracts, was found very sensitive to the presence of disease whether local or general in nature.   Lesions without apparent constitutional effects, a small blistered burn, a sterile incision, a tumor of pin-head size, produced distinct fluctuations of this value.   The

onset of rhinitis was indicated before the appearance of symptoms. The relation of clot retraction rate to the course of disease could be traced with particular assurity in the case of the tumor where a measure was also afforded of the progress of the lesion. Here, not only was the curve of retraction rate, in uncomplicated instances, nearly an exact reflection of the curve of tumor growth, but metastases were detected during or immediately following periods in the disease characterized by very low retraction rate readings. That reduction in the rate of clot retraction has to do with disease activity rather than with a poor state of bodily nutrition *per se*, is demonstrated by the fact that body weight was found to run parallel to retraction rate only in so far as it also reflected the stress of disease. Examples from the cases cited illustrate this: While in Text-fig. 11 body weight and clot retraction rate were simultaneously reduced during the active stages of tumor growth, in Text-figs. 9 and 10 the animals gained steadily in weight with the first inroads of the tumor, and retraction rate fell; in Text-fig. 14, when disease activity had totally ceased and cachexia was extreme, the rate of retraction remained at a normal level. There is also no consistent correspondence between anemia (hematocrit values) and defective clot retraction.

The alterations in blood composition that are responsible for changes in clot retraction have not been entirely explained. Hayem (12) showed that blood plasma deprived of all cellular elements formed a firm clot on standing and that the clot failed to retract. On the other hand, when he allowed the platelets alone to remain in the plasma, the clot which formed retracted normally. It was pointed out by Duke (13) that in purpura hæmorrhagica with a reduction in the number of platelets in the blood below about 50,000 per c.mm., specimens of blood formed non-retractile clots, and le Sourd and Pagniez (14), among others, have been able to induce clot retraction in such blood specimens by adding normal platelets. However, the latter authors (15) came to the conclusion, after both clinical and experimental observation, that, while the presence of blood platelets seems indispensable for normal retractility of the blood clot, retraction occasionally fails to take place even when platelets are present in abundance. This has been our experience, also, with regard to the relationship of both rate and extent of clot retraction to the blood

platelet content. It is possible also that the quality (fragility or chemical nature) of the platelets, as well as the quantity, may vary and play a rôle in clot retraction. The fact is well known, moreover, that the addition to the blood of minute traces of acid changes markedly the consistency of the clot, and the hydrogen ion concentration of the blood may readily be a determining factor in clot retractility.

SUMMARY.

Description is given of changes in blood coagulability found in diseases of the rabbit, including malignant tumor, spontaneous infections, non-bacterial diseases and lesions, and hemorrhagic states specifically induced. The changes involved variously the time of onset of blood coagulation, clot formation rate and the rate and extent of clot retraction.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

V. Production of Antihemotoxin by Immunization with Oxidized Pneumococcus Hemotoxin.

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INTRODUCTION.

It is known that active or reduced pneumococcus hemotoxin is antigenic since immunization with it induces a so called "antihemotoxin" which neutralizes the lysin (1). There is a marked lessening in the hemolytic activity of oxidized pneumococcus extracts (2), and it has seemed important to determine whether a loss in antigenicity accompanies the inactivation of the hemolytic property. Our investigation of this question has consisted in a comparison of the immunity response to the injection of reduced and oxidized pneumococcus extracts.

EXPERIMENTAL.

Methods.

Substances Injected.—A series of rabbits were immunized by injection of the following substances: (1) "reduced" or active pneumococcus extract which was stored in sealed tubes and subjected to a minimum exposure to air before injection; (2) oxidized or inactive extract which was exposed to air for 6 hours at 37°C. and then stored at 5°C.; (3) "vaccine" or heat-killed suspensions of pneumococci.

The reduced extract contained the active hemotoxin in high concentration, 0.001 cc. producing hemolysis when added to 2.5 cc. of red blood cells. The oxidized extract contained inactive hemotoxin and before each injection was always tested for the presence of traces of active lysin. That no significant amount of it persisted in the oxidized extract was proved by the fact that 0.3 cc. of the extract caused no detectable hemolysis when added to 2.5 cc. of blood cells. The results

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in animals injected with the heat-killed bacterial suspension were included for purposes of comparative study. The bacterial suspensions were heated at 56°C. for 30 minutes. Young cultures were used and the suspensions were heated at once to minimize autolysis and cell solution.

Immunization.—The reduced and the oxidized sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. to a maximum of 2.0 cc., in the last series of injections.

The "vaccine" or heat-killed bacterial suspension was also injected intravenously at the same time as the sterile cell solutions. Four courses of six daily injections of 0.1 cc. of a concentrated suspension of the bacteria were given so that the animals received in all the equivalent of bacteria from 24 cc. of culture.

All of the rabbits were bled on the 10th day after the last injection.

Titration of Antihemotoxin in Immune Serum.—The method used in determining the antihemotoxin value of the immune serum consisted in adding various amounts of serum to a constant amount of hemotoxin and determining the amount of serum required to completely neutralize the lysin. The hemotoxin-serum mixtures were shaken and then incubated for 45 minutes to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated to determine the presence of free or unneutralized hemotoxin. The detailed procedure is given below:

Constant "Dose" of Hemotoxin.—The hemotoxin content of a sterile pneumococcus extract was determined by a preliminary titration. Three times the amount of extract found sufficient to completely hemolyze 2.5 cc. of a 1 per cent suspension of washed rabbit cells was chosen as the test "dose" of hemotoxin. The pneumococcus extract was then diluted to such a concentration that 1.5 cc. of the dilute solution contained this amount of lysin.

Hemotoxin-Serum Mixtures.—0.5 cc. of different dilutions of serum was added to the series of tubes containing 1.5 cc. of the dilute hemotoxin solution. Owing to the inhibitory effect on the lysin of certain constituents of normal serum, control tubes were included containing the sera of the animals from bleedings made before immunization. The mixtures of hemotoxin and serum were shaken and then incubated for 45 minutes at 37°C.

Final Hemolysis Tests.—At the end of the 45 minutes, the presence of free or unneutralized lysin was determined by adding 0.5 cc. of a 5 per cent suspension of rabbit cells to the hemotoxin-serum mixtures. These final hemolysis test mixtures were incubated for 1 hour and readings of hemolysis made after centrifugation.

Control of Lysin "Deterioration" during Incubation of Hemotoxin-Serum Mixtures.—It was necessary to rule out the possibility that the lysin might be inactivated by oxidation since, should this occur, it might be confused with the immunological neutralization. For this purpose, control mixtures of 0.5 cc. salt solution plus 1.5 cc. of dilute hemotoxin were incubated with the series. At the end of the incubation period, these mixtures were diluted one-half and one-third; and the usual amount of red blood cells was added after these dilutions of the

control lysin had been made up to the correct volume of 2.0 cc. The control test mixture containing one-half and one-third the amount of lysin used, always showed complete hemolysis which proves that none of the apparent lysin neutralization or inhibition can have been due to inactivation of hemotoxin by oxidation during the preliminary incubation period.

Absence of Visible Precipitation in the Incubated Hemotoxin–Serum Mixtures.— Titrations of immune serum for the presence of antibodies neutralizing hemotoxins or enzymes are often complicated by the fact that the tested sera contain precipitins for other proteins present in the cell solutions. If during the preliminary incubation of the serum and cell solution, a precipitation or flocculation occurs, it is always possible that the active substance may be carried down with the precipitated particles. Subsequent tests of mixtures in which this precipitation has occurred indicate an apparent inhibition or neutralization of the test substance although in fact the active substance may have been only mechanically removed.

No such source of error existed in our experiments; for there was never any visible clouding or precipitation. The point is important since the immune sera contain a precipitin for the pneumococcus protein present in the bacterial cell solution used to furnish the hemotoxin. When the serum and bacterial solution are less dilute than in mixtures made according to the above procedure, protein precipitation occurs.

Strength of the Antihemotoxin in the Serum of Animals Immunized with Oxidized (Inactive) and Reduced (Active) Pneumococcus Hemotoxin.

In the following experiment a comparison was made of the hemotoxin-neutralizing power of the sera of animals immunized with oxidized (inactive) hemotoxin and that of sera immunized with the reduced (active) hemotoxin. The serum of a rabbit immunized with a heat-killed suspension of intact pneumococci was included for comparison. The general procedure has been described under Methods. In this experiment, the immune sera were tested against hemotoxin derived from pneumococci of the same fixed type (Type II) as the cell solutions used for immunization. The results are given in Table I.

The marked neutralizing power of the sera of animals immunized with both the oxidized (inactive) and the reduced (active) solutions of pneumococci is evident in Table I. The production of an antihemotoxin by the injection of cell solutions containing the active hemotoxin has been shown in Cole's (1) studies. The new fact revealed in Table I is the production of a neutralizing antibody by immunization with oxidized, that is to say, inactive pneumococcus hemotoxin. It is

evident that the serum of the animals injected with an oxidized extract which contains no demonstrable active hemotoxin possesses approximately the same neutralizing action as does that of animals immunized with the reduced, or active, hemotoxin.

The animal immunized with the heat-killed suspension did not produce antihemotoxin. This is an interesting correlation with the similar failure of immunization with properly prepared "vaccines" to yield an antibody for pneumococcus protein which like the hemotoxin is an endocellular substance (4).

TABLE I.

Antihemotoxin in Sera of Animals Immunized with Reduced (Active) Hemotoxin and Oxidized (Inactive) Hemotoxin.

No. of rabbit	Immunized by injection with	Hemolysis by previously incubated mixtures of hemotoxin and serum						
		Immune serum					Normal serum, before immunization	
		Amount of serum					Amount of serum	
		0.01 cc.	0.006 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.05 cc.	0.01 cc.
1	Inactive hemotoxin in oxidized cell solution	0	0	0	0	0	++++	++++
2	" "	0	0	0	+	±	+++	++++
3	Active hemotoxin in reduced cell solution	0	0	0	0	±	+++	++++
4	" "	0	0	0	0	0	+++	++++
5	"Vaccine" (heat-killed suspension of intact cells)	+++	++++	++++	++++	++++	++++	++++

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

In the preceding experiment, the neutralizing capacity of the immune sera was tested against hemotoxin derived from the homologous type of pneumococci. Cole (1) has shown that the antihemotoxin produced by immunization with the active substance is not type-specific,

but is effective against hemotoxin derived from all types of pneumococci. It seemed of interest to determine if the antihemotoxin produced by immunization with inactive (oxidized) hemotoxin is likewise equally effective against the hemotoxin derived from heterologous types of pneumococci. The protocol of an experiment testing this question is presented in Table II.

As shown in Table II the antihemotoxin which is produced by immunization with oxidized or inactive Pneumococcus is only species-

TABLE II.

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

Serum of animal immunized against	Hemolysis by previously incubated mixtures of hemotoxin and serum					
	Hemotoxin from Type I Pneumococcus		Hemotoxin from Type II Pneumococcus		Hemotoxin from Type III Pneumococcus	
	Amount of serum		Amount of serum		Amount of serum	
	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.
Inactive hemotoxin in oxidized pneumococcus cell solution (Type II)	0	0	0	0	0	0
Active hemotoxin in reduced pneumococcus cell solutions (Type II)	0	0	0	0	0	0
"Vaccine" (heat-killed suspension of intact cells)	++++	++++	++++	++++	++++	++++

specific and not type-specific since it neutralizes the hemotoxin derived from the cells of heterologous types of pneumococci. A species-specific antibody is apparently the usual type involved in the neutralization of bacterial hemotoxins; for the hemotoxin from cholera vibrios which are type-specific by agglutination, likewise gives rise to a common neutralizing antibody (3). The same holds true of the neutralizing antibody for the true toxins formed by diphtheria, tetanus and Welch bacilli, although each of these bacterial species includes strains which exhibit type specificity in tests with agglutinating immune sera.

Comparison of the Inhibiting Effect of Normal and Immune Serum upon Digitonin Hemolysis.

The serum of normal animals possesses a certain inhibitory action upon pneumococcus hemotoxin.   At least a part of this inhibition by normal or non-immune serum can be referred to lipoid constituents. In the case of tetanolysin, a number of investigations have proved that the increase in the neutralizing action of immune serum is not due to an increased concentration of the lysin-inhibiting constituents of normal serum, and it might be concluded by analogy that this is also true for the pneumococcus hemotoxin.   However, in view of the fact that the antihemotoxin described in the present paper is produced by immunization with a non-hemolytic antigen, it seemed desirable to determine whether the increased neutralizing capacity is due to a non-specific substance which would inhibit the hemolytic activity of other lytic agents.

Digitonin was chosen as the lytic substance to test this question, largely because hemolytically active digitonin is known to combine with cholesterol to form a non-hemolytic compound.   This reaction, in fact, is used as the basis of certain methods employed in the quantitative estimation of the cholesterol content of normal serum (4). The combination of cholesterol and digitonin is especially suited to a comparison of the degrees of neutralization of pneumococcus hemotoxin by normal and immune sera since Cole (1) has shown cholesterol to be one of the most important constituents involved in the inhibitory action of normal serum upon the pneumococcus lysin.

The experiments may be outlined as follows: The lytic action of a sample of digitonin was determined in the absence of serum.   With this information at hand two dilutions of digitonin were prepared in salt solution; one solution contained in 1.5 cc. twice the amount required to hemolyze 2.5 cc. of a 1 per cent suspension of rabbit cells; the other solution contained four times that amount of the lytic agent.   Mixtures of the digitonin with various dilutions of normal and immune rabbit serum were then prepared as described for the test mixture of pneumococcus hemotoxin and serum in the previous experiments.   After incubation for 1 hour at 37°C. to allow time for the combination of digitonin with the cholesterol and other serum constituents, 0.5 cc. of a 5 per cent suspension of rabbit cells was added.

The results of these experiments were conclusive. Although relatively large amounts of both the normal and immune serum inhibited the digitonin hemolysis, there was no significant difference in the action of the two. The sera obtained by immunization with both the reduced and oxidized solutions exhibit more than 100 times the neutralizing capacity of normal serum when tested against pneumococcus hemotoxin, yet they exhibited no greater neutralizing or inhibiting action on digitonin hemolysis than did this serum. From the results, it is obvious that the neutralizing antibody produced during immunization with the hemolytically inactive, oxidized hemotoxin is distinct from the usual lysin-inhibiting constituents of normal serum.

Neutralization Experiments with Pneumococcus Immune Serum and Hemotoxins of Other Bacteria.

Experiments were conducted to determine whether the immune sera which neutralize pneumococcus hemotoxin have any neutralizing or inhibiting action upon the hemotoxins of other bacteria. Tetanolysin and the lysin of the Welch bacillus were the hemotoxins tested. Normal serum and the serum produced by immunization with heat-killed pneumococcus vaccine were included as controls, since certain constituents of normal serum have a marked inhibitory action upon the lysins of both of these anaerobic bacilli. The procedure employed was essentially the same as in the previous experiments with the pneumococcus lysin. The constant "dose" of tetanolysin and Welch lysin used in these experiments represents 8 to 10 times the amount of lysin required for the hemolysis of 2.5 cc. of rabbit cells in the absence of serum. A larger "dose" of lysin was chosen than in the previous experiments because these lysins are inhibited to a greater extent by normal serum than is the lysin of pneumococci. The preparation of serum-hemotoxin mixtures and other steps in the procedure were the same previously described.

Three normal sera and the sera of the four rabbits immunized with oxidized and with reduced pneumococcus extracts were included in the experiment. The results of tests upon tetanolysin with a typical normal and a typical immune serum are presented in Table III.

In spite of the markedly increased capacity of the immune serum to

neutralize the hemotoxin of Pneumococcus, the results presented in Table III which were typical for the experiments show that immunization of animals with this hemotoxin does not increase the ability of their serum to neutralize or inhibit the hemotoxin of the tetanus bacilli.

The results of the tests with the Welch lysin were not so clean-cut, since they were complicated by marked differences in the neutralizing or inhibiting capacity of different normal rabbit sera—a fact previously observed in another investigation (5). The sera of the various immune rabbits also showed marked differences among themselves in their inhibitory action on the Welch lysin. However, a survey of the results as a whole indicates quite clearly that the sera of rabbits

TABLE III.

Inhibition or Neutralization of Tetanolysin by Pneumococcus Immune Serum and by Normal Serum.

Serum	Hemolysis by previously incubated mixtures of serum and tetanolysin		
	Amount of serum		
	0.005 cc.	0.001 cc.	0.0002 cc.
Rabbit immunized against pneumococcus hemotoxin....................................	0	+	+++
Normal rabbit....................................	0	0	++

immunized against the hemotoxin of Pneumococcus exerted no greater neutralizing or inhibiting effect upon the lysin of Welch bacilli than was found with the serum of normal or non-immune animals.

COMMENT.

The preceding experiments indicate that a neutralizing antibody may be produced by immunization with the hemolytically inactive hemotoxin present in oxidized solutions as well as by immunization with the active hemotoxin. The antibody is a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci. It seems to be without effect upon the hemotoxins of tetanus and Welch bacilli.

The theoretical and practical importance of "toxoid immunization"

gives a special significance to the above results. But the data thus far procured do not enable one to state that the inactive or oxidized lysin possesses antigenic properties identical with those of the reduced or active hemotoxin. A more complete discussion of the antigenic properties of the inactive oxidation products of pneumococcus hemotoxin will be presented in a subsequent paper.

SUMMARY.

Immunization with solutions of the intracellular substances of pneumococci, in which the hemotoxin has been rendered inactive by oxidation, yields an antibody which neutralizes the active or reduced hemotoxin.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

I. The Effects of Partial Parathyroidectomy.

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Observations on the endocrine tissues of rabbits irradiated with a quartz mercury arc lamp have disclosed the striking hypertrophy of the parathyroid glands produced by the irradiation (1). Histologically, this hypertrophy was found to be a true hyperplasia of the endocrine tissue, but a study of the calcium and inorganic phosphate content of the blood of these rabbits failed to reveal any significant changes from the normal levels (2).

That parathyroid integrity is essential to the maintenance of a normal blood calcium level has been established by the extirpation experiments of many investigators, but the upper limit of calcium concentration seems to be determined by factors independent of parathyroid control. Hence, in the normal animal, parathyroid hypertrophy due to an external stimulus, such as light, appears to result only in an increase in the factor of safety, and to be unaccompanied by a recognizable increase in functional activity for which there is no physiological demand.

On the other hand, the presence of an increased factor of safety might be revealed by an unusual functional strain or emergency, and a study of such conditions, experimentally produced, might throw further light upon normal parathyroid function. Having at hand methods of producing a hypertrophic condition of the parathyroid glands by means of ultra-violet light, and a condition of reduced function by partial extirpation, we undertook a series of experimental comparisons of groups of rabbits with enlarged, normal, or partially extirpated parathyroids.

In preparation for these studies, partial parathyroidectomies were performed on a number of rabbits, some normal, some after irradiation with ultra-violet light. The effects of the operation itself upon the calcium and the inorganic phosphate level of the blood of these animals form the subject matter of this paper.

Experimental Procedure.

Since the partially parathyroidectomized rabbits were to be used subsequently in other experiments, the operations were performed on small groups of animals and at various seasons of the year. In some instances only the two external parathyroids were removed; in others, the thyroid gland was divided at the isthmus, and one lobe, with its internal parathyroid gland, was removed also. The animals were all healthy, adult, albino males, and the glands were removed aseptically, under ether anesthesia, after a median incision and blunt dissection of the tissues of the neck. The operative wounds healed without infection. At intervals after the operation, small samples of blood were taken from the marginal ear vein and allowed to clot, and calcium (3) and inorganic phosphorus (4) determinations were made on the serum.

Preliminary observations on two normal rabbits, after removal of the two external parathyroid glands, showed that the operation was followed by a sharp drop in blood calcium to a low level on the 2nd or 3rd day, and a more gradual increase toward the normal over a period of days or even weeks. No signs of tetany appeared in these two animals.

Experiment 1.—The next groups of six and four normal rabbits respectively were deprived of both external parathyroid glands and one internal gland also. In spite of individual variations in reaction, the trends of the blood calcium curves of these rabbits were essentially similar. Immediately after operation, the blood calcium of all ten rabbits dropped from normal levels, between 11.6 and 13.0 mg. per 100 cc. of serum, to 6.2 to 10.2 mg. (37 to 88 per cent of the control figures) by the 2nd day. The rabbit with 6.2 mg. of calcium per 100 cc. of blood serum succumbed on that day in severe tetany. From this point the blood calcium of seven of the remaining nine rabbits began to rise towards normal, so that figures of 10.8 mg. to 12.6 mg. were obtained on the 4th to the 6th days, and these levels were usually maintained thereafter (Chart 1). The other two rabbits had persistently lower blood calcium levels, and one of them showed a drop from 8.5 mg. per 100 cc. of serum on the 14th day after operation, to 4.8 mg. on the 17th day, when it had a typical tetanic seizure and died. The other rabbit maintained a serum calcium level between 8 and 10 mg. for 35 days. 2 days later it was transferred to a fasting

experiment, in which it succumbed on the 5th day. On the day before death its blood calcium was 5.65 mg. per 100 cc. of serum.

The inorganic phosphorus in the blood serum of most of these rabbits was not examined at regular intervals. It was observed, however, that before death the serum phosphorus of the rabbits that succumbed on the 2nd, 17th, and 42nd days after operation was 9.4 mg., 13.1 mg., and 12.0 mg. respectively per 100 cc. of serum, as compared with a normal average of 7.4 mg.

In this experiment the capacity of the residual parathyroid tissue to respond to sudden demands is shown by the fact that in seven of ten rabbits, after the initial drop, blood calcium levels only 5 to 10 per cent below normal were attained within a week, and these levels were usually maintained thereafter. If, after partial para-
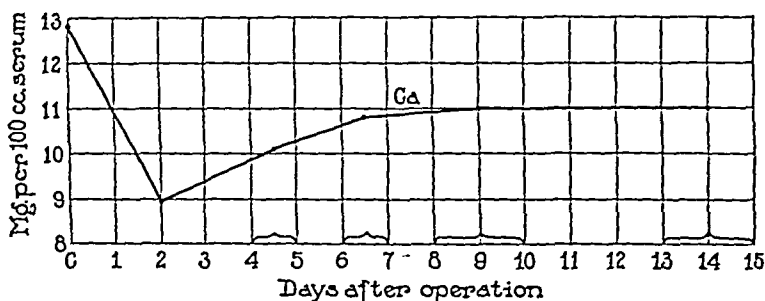

CHART 1. Experiment 1. Serum Ca levels after partial parathyroidectomy.

thyroidectomy in normal rabbits, the remaining tissue is usually able to restore the blood calcium level nearly to normal in 4 to 6 days, this is the interval in which evidence of parathyroid hyperplasia and consequent increase in the factor of safety must be sought in irradiated rabbits.

In the following experiment, normal and irradiated rabbits were partially parathyroidectomized and observed during the ensuing period.

Experiment 2.—A group of ten adult albino rabbits had both external parathyroids removed. Five of these animals, with backs clipped and ears shaved, had been exposed for 30 minutes a day, 6 days a week, for 3 weeks, at 1 meter distance, to a quartz mercury arc lamp (67 volts, 5.5 amperes). These rabbits presumably had hypertrophic parathyroid glands (1). The exposures were continued for the further period of observation. The five other rabbits served as controls for the

irradiated group. All were bled for a serum sample before operation, on the 1st, 2nd, and 4th days following, and then every 2 or 3 days to the 15th or 16th day.

A difference in the reaction of the irradiated and control rabbits to the loss of their external parathyroids became apparent within 24 hours after operation. None of the irradiated rabbits showed signs of tetany, but three of the controls were observed in acute attacks, characterized by gross and fibrillar twitchings of the voluntary muscles,—notably those of the jaw,—tonic and clonic convulsions, opisthotonos, deep, heaving respiration, venous engorgement, and
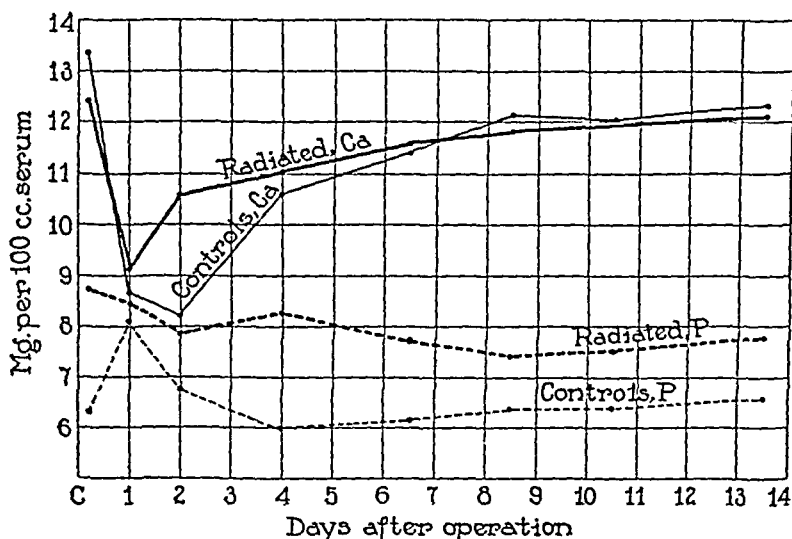

CHART 2. Experiment 2.   Comparison of serum Ca and P in irradiated and control rabbits after partial parathyroidectomy.

excessive salivation. In two of these rabbits an intravenous injection of 10 cc. of $\frac{x}{8}$ CaCl₂ relieved the signs immediately, but one of them succumbed during the following night. The third rabbit recovered spontaneously.

The absence of all signs of tetany in the five irradiated rabbits led us to infer that the hyperplasia of the internal glands produced by the radiations had increased their functional capacity, *i.e.*, their factor of safety, to a degree sufficient to protect the animals. This increase in the factor of safety in the irradiated rabbits is reflected in a comparison of their blood calcium and inorganic phosphorus

curves. In both groups the fall in calcium was immediate, so that 24 hours after operation the average for the irradiated rabbits had dropped from 12.4 to 9.1 mg. (73 per cent of normal), and for the controls from 13.4 to 8.7 mg. (65 per cent of normal) per 100 cc. of serum. But the following day the blood calcium average in the irradiated group rose to 10.6 mg. per 100 cc. of serum, while the four survivors of the control group suffered a further loss to 8.2 mg. By the 7th day this difference had been overcome, and both groups then maintained calcium levels approaching normal (Chart 2). Similarly, due to sharp rises in the blood phosphorus of the three control rabbits

TABLE I.

Blood Calcium and Inorganic Phosphorus 1 Day after Partial Parathyroidectomy.

Normal rabbits				Irradiated rabbits			
No.	Calcium	Phosphorus	Ratio $\frac{Ca}{P}$	No.	Calcium	Phosphorus	Ratio $\frac{Ca}{P}$
	Mg. per 100 cc. serum				Mg. per 100 cc. serum		
18	10.9	6.0	1.82	13	10.3	8.5	1.21
19	9.5	4.8	1.96	14	10.1	7.2	1.40
20*	8.5	8.7	.98	15	8.9	8.6	1.03
21†	8.0	11.0	.73	16	8.5	8.7	.98
22*	6.0	9.9	.61	17	7.8	9.2	.85

* Observed in tetany.
† Died.

that had outspoken tetany, the average for the group was carried upward on the 1st day from 6.3 mg. to 8.1 mg. per 100 cc. of serum. Meanwhile, in the irradiated group, with a high average blood phosphorus to start with (2), only one of the rabbits showed a further rise in blood phosphorus following the operation. This rabbit had the greatest drop in calcium of any in the irradiated group.

Such a reciprocal relationship between blood calcium and phosphorus, pointed out by Howland and Kramer (5), and recently observed in normal dogs by Mayerson, Gunther, and Laurens (6), is illustrated in this experiment by arranging the rabbits of each group in the order of their blood calcium levels on the day following partial parathyroidectomy (Table I). In both groups the rabbits

with the lower calcium values after partial parathyroidectomy had the higher figures for blood phosphorus.

A final group of five normal rabbits, whose external parathyroid glands were extirpated, confirmed the earlier observations by exhibiting an average fall of blood calcium from 12.0 mg. to 8.8 mg. per 100 cc. of serum, 24 hours after operation. In three of these rabbits the calcium level was restored to normal in 7 to 9 days. The other two had persistently lowered blood calcium, and one rabbit, after transfer to a fasting experiment, on the 9th day, suffered a further drop in Ca to 4.9 mg. and died on the 16th day after the partial parathyroidectomy. 2 days before death this rabbit's blood
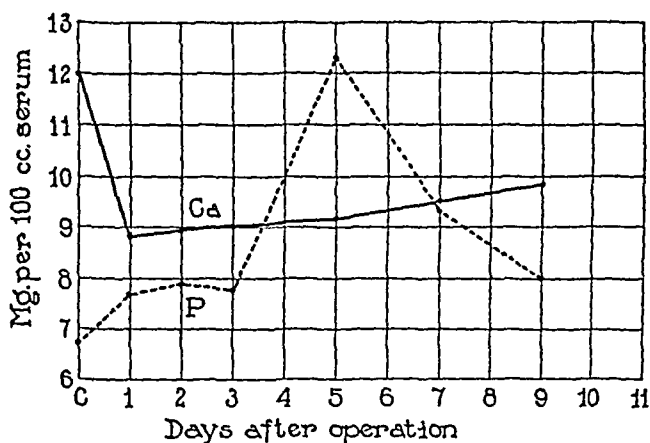

CHART 3. Serum Ca and P levels after partial parathyroidectomy.

phosphorus was 12.68 mg. per 100 cc. of serum. All the rabbits in this group exhibited a high blood phosphorus at one time or another after operation, the average for the group on the 5th day being 12.3 mg. per 100 cc. of serum, compared with a control level before operation of 6.7 mg. (Chart 3).

That the ether anesthesia and the operative procedure did not contribute to the fall in serum calcium, or the rise of inorganic phosphorus, was shown by the absence of such changes in two control rabbits, in which identical operations were performed, except for the removal of the parathyroid glands.

The Relative Significance of a Fall in Blood Calcium and of a Rise in Blood Phosphorus in Parathyroid Tetany.

We have already noted the marked rise in the inorganic phosphorus of the blood serum, which often accompanied the drop in serum calcium after partial parathyroidectomy. A rise in inorganic phosphorus occurred in some rabbits which did not develop tetany, but it was never absent in those that did, and was apparently associated with the onset of the acute attack. The question arises whether it is only the fall in calcium, or in reality the accompanying change in the ratio of calcium to phosphorus in the blood serum that is more significant in tetany.

TABLE II.

Rabbit No.	Onset of tetany day after operation	Ca	P	Ratio $\frac{Ca}{P}$	Outcome
		Mg. per 100 cc. serum			
4	17th	4.8	13.1	.37	Died
6	41st	5.7	12.0	.48	Died
9	2nd	6.2	9.4	.66	Died
21	1st	7.9	11.0	.72	Given CaCl₂; died
20	1st	8.5	8.7	.98	Mild attack; recovered
22	1st	6.0	9.9	.61	Given CaCl₂; recovered
26	16th	4.9	12.7	.38	Died
Average..........		6.3	11.0	.60	

Normally, in rabbits, this ratio $\frac{Ca}{P}$ is not less than 1, and it usually lies between 1.3 and 2.6. Among 103 normal rabbits, with a calcium level, on admission, between 11.1 and 13.9 mg. per 100 cc. of serum, the lowest ratio $\frac{Ca}{P}$ was 1.10 and the three highest, with one exception, were 3.3. The exceptional rabbit had a very low serum phosphorus (1.8 mg.) and a unique ratio $\frac{Ca}{P}$ of 7.30. The average ratio for all the rabbits was $\frac{12.4}{6.4} = 1.9$.

In the rabbits that developed acute tetany after partial parathy-

roidectomy, on the other hand, the ratio $\dfrac{Ca}{P}$ was changed not only by the fall in calcium, but by an abnormal rise in phosphorus also. Table II shows the ratio $\dfrac{Ca}{P}$ in these rabbits, according to the last figures obtained before the attack, and it will be noted that high phosphorus figures contribute as much to change the $\dfrac{Ca}{P}$ ratio from normal, as do the low figures for blood calcium.

Of nine other partially parathyroidectomized rabbits, which were not observed in tetany, four at one time or another had calcium

TABLE III.

Rabbit No.	No. of days after operation	Lowest calcium level	Phosphorus level	Ratio $\dfrac{Ca}{P}$
		Mg. per 100 cc. serum		
3	2	8.6	7.7	1.12
5	2	7.6	8.6	.88
7	18	10.6	5.7	1.9
19	2	8.4	5.6	1.5
18	1	10.9	6.0	1.82
8	1	9.2	8.0	1.15
10	1	9.6	10.8	.89
11	5	7.3	12.2	.60
12	3	6.6	8.1	.81

and phosphorus levels comparable with some of those in the tetanic animals. The other five did not have either very low calcium or high phosphorus at any time after operation, and their $\dfrac{Ca}{P}$ ratios were usually more than 1 (Table III).

DISCUSSION.

Our observations are in accord with those of other investigators who have studied calcium and phosphorus metabolism after parathyroidectomy. The characteristic drop in blood calcium has often been found and needs no further comment. This fall in blood calcium is certainly a primary reaction, which may or may not be followed by a

rise in phosphorus, either immediately, or later, when the blood calcium is returning toward the normal level.

A phosphorus retention, following parathyroidectomy in dogs, was observed in 1911 by Greenwald (7), who has since published a series of papers in which the work of others is reviewed. He has recently stated (8, a) that in over 50 parathyroidectomized dogs tetany never appeared without phosphorus retention, and conversely, a drop in phosphorus excretion after attempted parathyroidectomy was observed in only one of ten dogs that did not develop tetany. However, the increase in "acid-soluble" phosphorus in the blood of parathyroidectomized dogs in tetany, which Greenwald had observed in 1913 (8, b), was not so marked in his later experiments, and in 1924 (8, c) he appears to minimize its significance, assuming that the excess of phosphates retained is deposited in the tissues, and only reappears in the circulation under certain abnormal conditions, such as after ether anesthesia, or cerebral or general anemia. In those of our rabbits that developed tetany, an excess of phosphorus was found in the blood.

Salvesen (9) also found that the inorganic phosphorus of the blood serum of completely parathyroidectomized dogs was considerably increased, and tended to remain above the normal value. According to one protocol, a dog with a normal blood calcium of 10 mg. per 100 cc. of serum and inorganic phosphorus of 3.0 mg. $\left(\dfrac{Ca}{P} = 3.3\right)$ developed severe tetany 2 days after complete parathyroidectomy, with a $\dfrac{Ca}{P}$ ratio of $\dfrac{3.50}{12.5} = 0.28$, and the serum phosphorus level remained above 6.0 mg. per 100 cc. of serum thereafter.

In a study on blood changes and clinical symptoms, following oral administration of phosphates in dogs, Salvesen, Hastings, and McIntosh (10) discuss the changes in blood phosphorus and calcium that result, and they conclude that the fall in calcium is the significant factor in the development of tetany.

In a recent private communication, Dr. John Howland stated his conviction that the clinical syndrome known as infantile tetany is certainly not the result of a high phosphorus content of the serum which drives down the calcium concentration (11), and observed that infantile tetany is found at times with a very low phosphorus concentration of serum. He has found that in many instances the phosphorus is high, perhaps in two-thirds of the cases, but there is the one-third which cannot be neglected.

We shall postpone further discussion of the subject until we report a series of observations on rabbits to which secondary sodium orthophosphate was given subcutaneously. The point to be noted at this time is that the calcium level itself was apparently not the sole determining factor in the development of tetanic signs in these partially

parathyroidectomized rabbits.   Only in connection with an increase in blood phosphorus, which further disturbed the normal $\dfrac{\text{Ca}}{\text{P}}$ ratio, were signs of hyperirritability observed.

SUMMARY.

Previous irradiation of rabbits with ultra-violet light, with a consequent hypertrophy of the parathyroid glands, resulted in some degree of protection to these animals, when part of the parathyroid tissue was extirpated.   This fact indicates that the remaining hyperplastic tissue was potentially active, and that the increased factor of safety represented by this condition of the remaining tissue resulted in less loss of blood calcium, a more rapid initiation of recovery, and an absence of the rise in the inorganic phosphorus of the blood serum which was a characteristic result of partial parathyroidectomy in normal rabbits.   A fall in calcium in all the irradiated rabbits, without a corresponding rise in phosphorus, shows that the drop in calcium is the primary reaction, and a rise in phosphorus a secondary reaction following parathyroidectomy.   But the relation of the rise in phosphorus to the development of tetany remains obscure.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROID-ECTOMIZED RABBITS.

II. The Effects of Injections of Calcium Chloride or of Disodium Hydrogen Phosphate.

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Although the calcium and inorganic phosphorus levels of the blood serum of rabbits appear to be more affected by seasonal changes in environment and by caging (1) than by the hypertrophy of the parathyroid glands induced by ultra-violet radiations (2), the hypertrophic changes in the glands are evidence of an increased "factor of safety" which is manifest in the reactions of irradiated animals to partial parathyroidectomy (3). A comparison of their behavior with that of normal rabbits subjected to an identical operation[1] led us to a further investigation of the reactions of irradiated, normal, and partially parathyroidectomized rabbits to other experimental procedures, in order to test the functional capacity of their parathyroids. The three groups of rabbits were assumed to differ in the relative amount and potential activity of their parathyroid tissue, the irradiated animals having hypertrophic glands and the partially parathyroidectomized rabbits having only so much parathyroid tissue as was necessary for the maintenance of a blood calcium level approaching normal.

Our first observations on the effects of the injection of calcium chloride or of disodium hydrogen phosphate into irradiated, normal, and partially parathyroidectomized rabbits gave some unexpected results, and turned our immediate attention from the primary object of the investigation to an interest in the rôle of inorganic phos-

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[1] All operations for removal of parathyroids were performed under full ether anesthesia.

phates in the development of outspoken clinical tetany, under con-
ditions of lowered blood calcium.

Calcium Injections.

Two experiments were performed, the first with four rabbits;
the second with seven.

Experiment 1.—Two normal, adult, male rabbits served as controls. The other
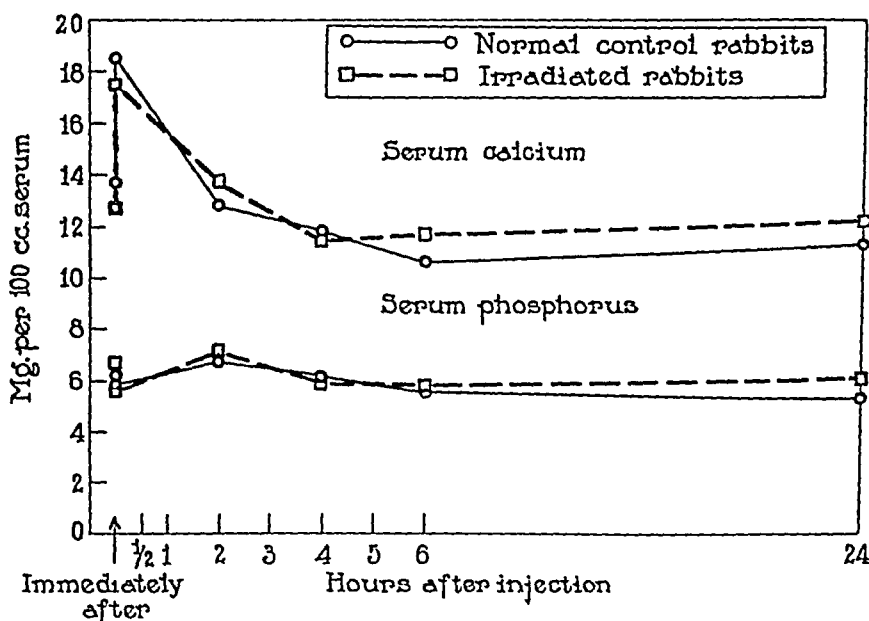two, with backs and ears closely clipped, had been exposed daily for 30 days to the

CHART 1. Experiment 1. Serum Ca after injection of CaCl₂ in normal and
irradiated rabbits.

total radiations of a quartz mercury arc lamp (67 volts, 5.5 amperes), at 1 meter's
distance. During this period the daily exposures were increased from 10 minutes
to 2 hours. Then all four rabbits received an intravenous injection of sterile $\frac{M}{8}$
CaCl₂ solution, representing 25 mg. of Ca per kilo body weight. The volume of
fluid was about 10 cc. Blood samples were taken before and immediately after the
injections, and 2, 4, 6, and 24 hours later, and calcium (4) and inorganic phosphorus
(5) determinations were made on the serum.

Chart 1 shows that the reactions of the normal and the irradiated
rabbits were essentially similar. The excess calcium practically

disappeared from the blood in less than 2 hours, and the calcium level subsequently fell somewhat below that of the control specimens. This dose of calcium chloride had only a relatively slight effect upon
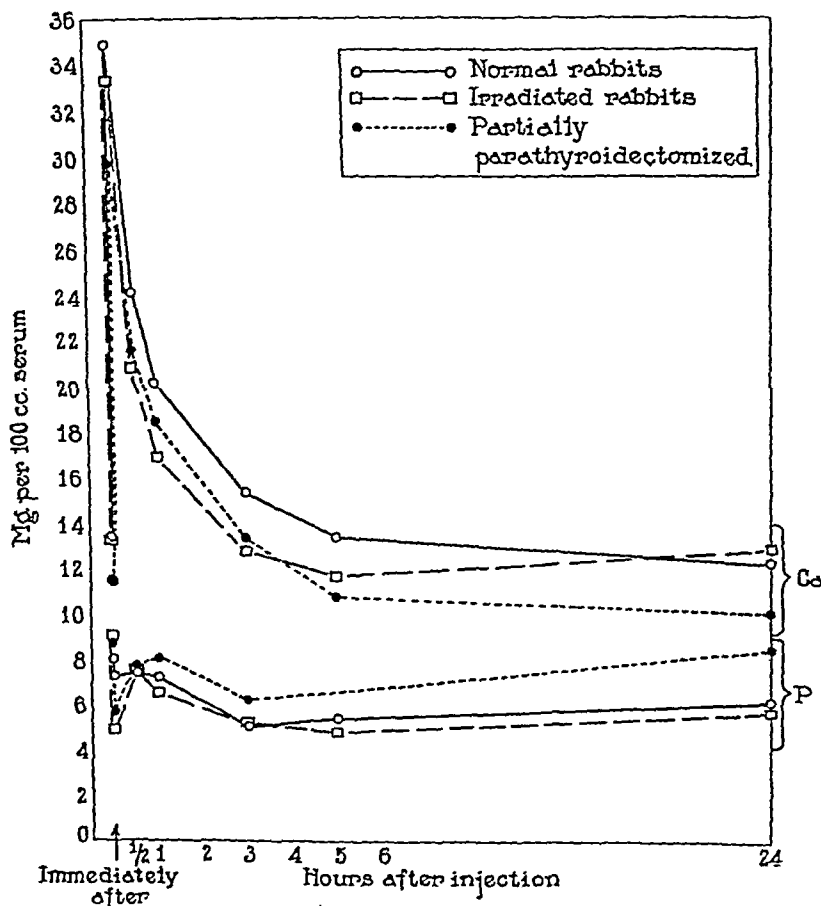

CHART 2.   Experiment 2.   Serum Ca after injection of CaCl₂ in normal, irradiated, and partially parathyroidectomized rabbits.

the inorganic phosphate levels. The initial drop is due partly to dilution of the blood serum. A reciprocal relationship is seen in the slight rise during the rapid drop in calcium.

Experiment 2.—In this experiment two normal and two irradiated rabbits were supplemented with three partially parathyroidectomized animals. Two of these had been deprived, 6 weeks before, of both external parathyroids and one-half the thyroid with its internal parathyroid gland. The other rabbit had had its external parathyroids removed 4 weeks previously. During these intervals the serum calcium of these rabbits had been gradually restored to 11.4, 12.0, and 11.2 mg. respectively, indicating a corresponding restoration of parathyroid function.

These rabbits received intravenous injections of $CaCl_2$ in sterile $\frac{N}{4}$ solution, in doses of 35 to 50 mg. of Ca per kilo of body weight. Because of the increased concentration, the total volume injected was somewhat less than in Experiment 1. Small blood samples were withdrawn from an ear vein, just before and after injection, and 30 minutes, 1, 3, 5, and 24 hours later. The results of Ca and P determinations on these specimens are shown in Chart 2.

In all of these rabbits the immediate rise of the blood Ca to a high level was followed by a rapid disappearance of the excess from the blood, so that the control levels were restored in 3 to 5 hours. No striking differences were observable between the average values for the three groups, and indeed the individual figures obtained 30 minutes and 1 hour after injection were remarkably similar throughout. Apparently the condition of the parathyroid glands had little influence on the disappearance of excess calcium from the blood stream. It is interesting to observe in the partially parathyroidectomized rabbits that the blood calcium returned to its former lower level, as Salvesen (6) and others have observed repeatedly under similar conditions.

Thus the calcium level of the blood seems to be determined by a balance between parathyroid activity and other factors independent of parathyroid control. When there is a reduction of parathyroid activity, as after partial parathyroidectomy, the calcium level drops correspondingly. But even an excess of parathyroid tissue does not maintain an excess of calcium already present in the blood, in opposition to the forces which tend to restore it to a normal level (2).

That the immediate reduction of inorganic phosphorus was due in part to the calcium content of the injection fluid is shown by the greater fall in phosphorus in Experiment 2, as compared with Experiment 1, in which a larger volume of fluid was injected. This drop was most marked in the irradiated rabbits of both series, in which an average fall from 7.83 mg. to 5.32 mg. per 100 cc. of serum occurred. The fall in phosphorus was as temporary as the cor-

responding rise in calcium and illustrates again the tendency to reciprocal reactions which several authors have already pointed out. The experiment does not indicate whether the restoration of inorganic phosphorus in the first half-hour after the calcium injection was a cause or an effect of the correspondingly rapid fall in calcium.

Phosphate Injections.

Subcutaneous injections of sterile disodium hydrogen phosphate (Na_2HPO_4) were given in $\frac{M}{2}$ solution in doses of 100, 125, or 150 mg. of P per kilo body weight.

Experiment 3.—The ten rabbits injected with 100 mg. P per kilo reacted in an essentially similar manner. Five were normal, three had been partially parathyroidectomized (two external glands and one-half the thyroid extirpated 16 to 28 days previously), and two had been irradiated daily for 23 or 26 days in increasing periods of 10 to 90 minutes at 1 meter's distance from the quartz mercury arc lamp. Blood samples withdrawn from an ear vein before the injection, and 4, 6, 12, and 24 hours afterwards were allowed to clot and the sera were analyzed for Ca (4) and inorganic P (5).

The average figures for each group closely represent the individual findings. These averages, plotted in Chart 3, show, in the first place, the somewhat lower blood calcium of the partially parathyroidectomized rabbits before the injection, and a relatively high blood phosphorus in both irradiated (3) and partially parathyroidectomized animals (7). No significant differences occurred in the reactions of the normal and the irradiated rabbits, and the partially parathyroidectomized animals differed from the others only in the fact that the lower calcium level which distinguished them was maintained throughout the period of observation. The subcutaneous injection of disodium hydrogen phosphate was followed by a prompt rise of inorganic phosphorus in the blood. The peak of the rise, as the next experiment will show, probably occurred before the 4 hour blood sample was taken. In all of these rabbits the excess phosphorus in the blood was practically eliminated within 12 hours. In fact, the most striking feature of the curves is the drop in the blood phosphorus of the irradiated and the partially parathyroidectomized rabbits well below the high levels found in their blood before the injection.

Since the blood is normally a supersaturated solution of calcium bicarbonate and calcium hydrogen phosphate (8), it is to be ex-
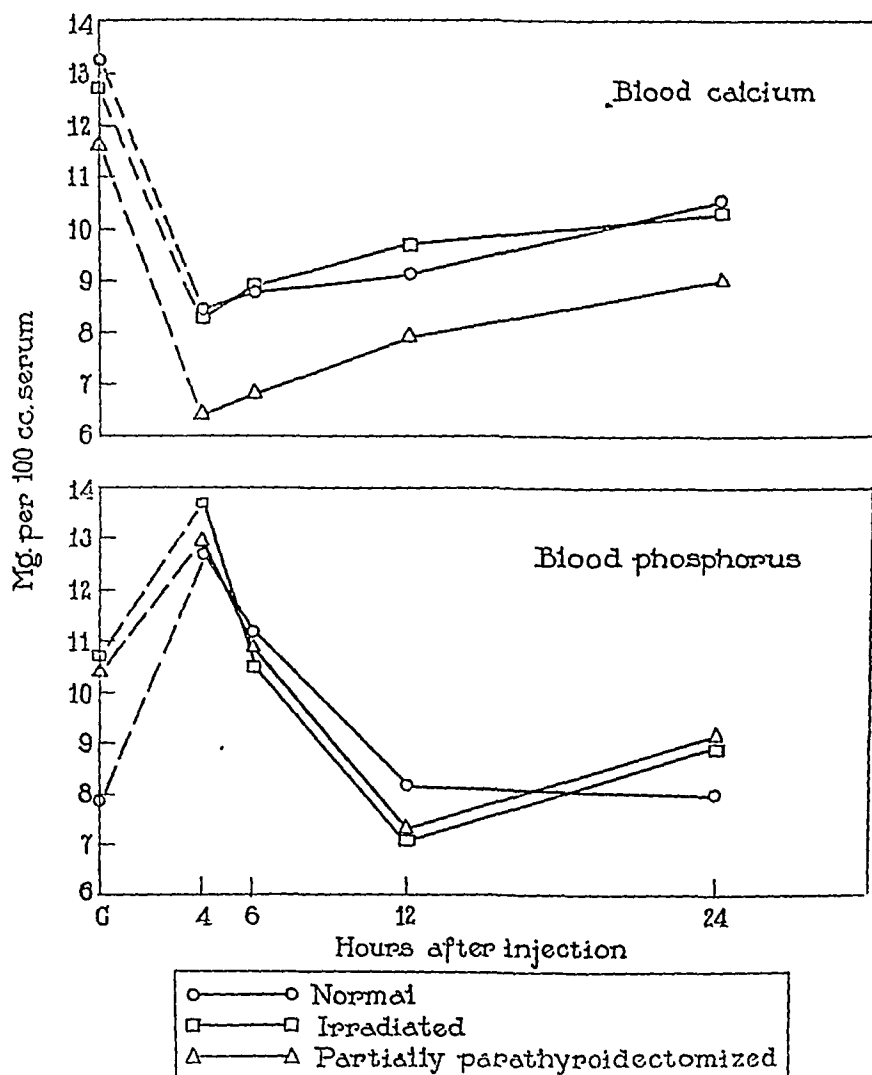

CHART 3. Experiment 3. Serum Ca and P after injection of Na_2HPO_4 in normal, irradiated, and partially parathyroidectomized rabbits.

pected that a rise in other phosphates would cause a fall in calcium. Binger (9) produced tetany in dogs by phosphate injections and found the blood calcium reduced beyond the degree attributable

to serum dilutions. Salvesen, Hastings, and McIntosh (10) have recently raised the blood phosphorus level in dogs by oral administration of mono- and disodium orthophosphates, and observed a corresponding fall in blood calcium.

In this experiment the blood calcium level fell promptly in all the rabbits, probably reaching its nadir about the 4th hour, and then rising slowly toward normal. Some scattered observations after 48 and 72 hours indicate that the normal levels were attained within that time. The lowest Ca level recorded was 6.0 mg. per 100 cc. serum, found 4 hours after the phosphate injection in a partially parathyroidectomized rabbit. This rabbit showed no outspoken tetany, but another partially parathyroidectomized animal with a Ca level of 6.4 mg. had a single convulsive seizure 6 hours after injection. All of the rabbits were restless and hypersensitive, but no other acute attacks of tetany were observed.

Although they were studied in small groups, all the rabbits that received subcutaneous injections of $\frac{M}{2}$ disodium hydrogen phosphate, representing 125 or 150 mg. of P per kilo body weight, may be considered together, for the size of the dose does not serve to distinguish their reactions, which depended rather upon the calcium and phosphorus levels attained in the blood.

Experiment 4.—Nineteen rabbits in four groups are included in this experiment: (1) four rabbits were normal, adult males; (2) four rabbits had been irradiated, three of them for 15 to 25 minutes 6 days a week for 26 to 30 days, at a distance of 50 cm. from a quartz mercury arc. The other had been exposed 10 to 90 minutes a day for 26 days at 1 meter's distance; (3) five rabbits had been irradiated with the quartz mercury arc (30 minutes daily, 18 to 21 days, at 1 meter's distance), and then partially parathyroidectomized with the removal of the two external parathyroids only. Irradiation was continued for the 13 to 16 days between the operation and the date of the phosphate injections; (4) six rabbits had not been irradiated, but underwent similar operations 13 to 16 days before the experiment. Disodium hydrogen phosphate in $\frac{M}{2}$ solution was given to all these rabbits subcutaneously in single doses, representing 125 to 150 mg. P per kilo body weight. Blood samples taken before and at intervals after the injections were analyzed for Ca and inorganic P.

In all nineteen rabbits the immediate results of these phosphate injections were a rise in blood phosphorus, a drop in the blood calcium level, and the onset of signs and behavior characteristic of

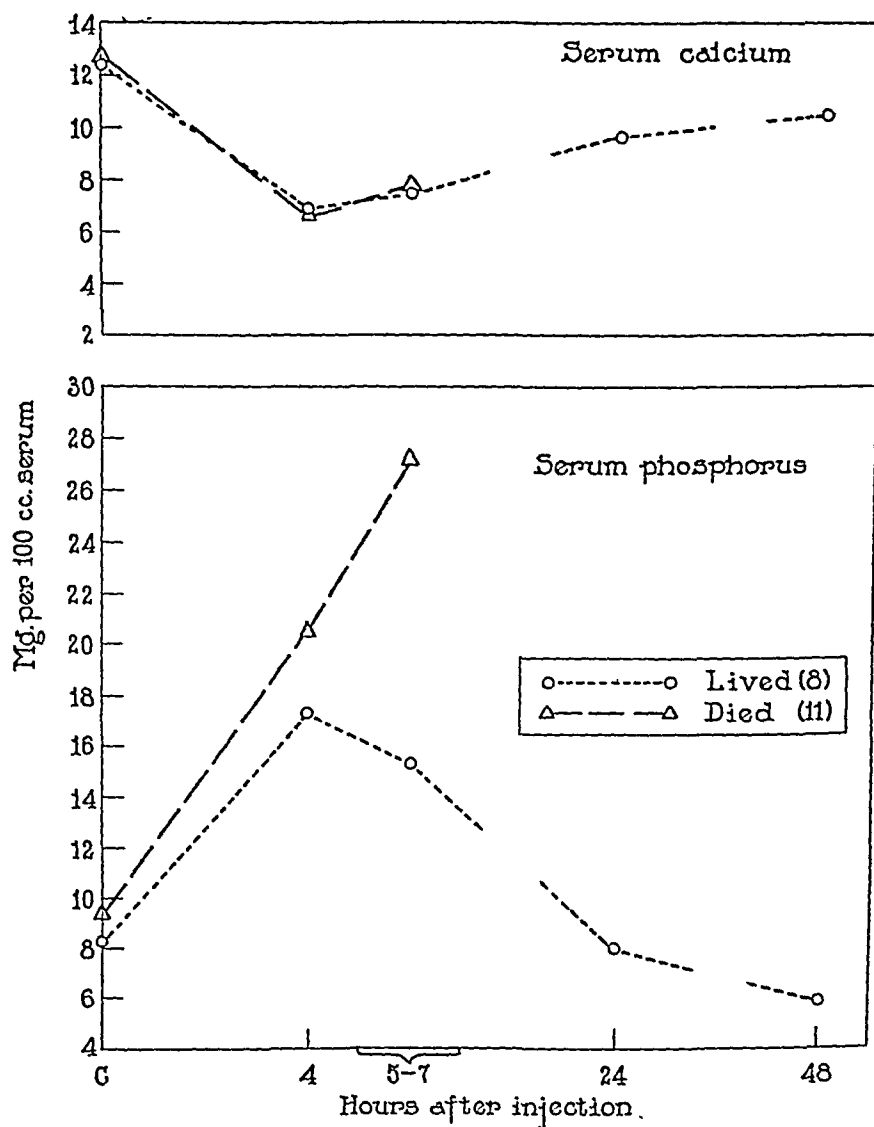acute tetany. The reaction usually was most severe between the 4th and 8th hours, an interval in which ten rabbits died.   Another

CHART 4. Experiment 4. Serum Ca and P after injection of Na_2HPO_4 showing association of P retention and death.

rabbit was found dead the following morning. The other eight, after exhibiting varying degrees of tetany, survived and returned to a state of normal irritability in 24 to 48 hours.

As already stated, the object of these experiments was to observe possible differences in the behavior of irradiated, normal, and partially parathyroidectomized rabbits to sudden changes in the calcium and phosphate balance in the blood. As in the first three experiments, so in this one a review of the rabbits' reactions shows that they cannot be distinguished according to the apparent state of the animal's parathyroid glands. Equal numbers lived and died in each group, except that among the irradiated, partially parathyroidectomized rabbits one lived and four died. Possibly these animals were more susceptible than those in the other groups. On the whole, the experiment failed to show differences in reaction which might be referred to differences in parathyroid function due to irradiation or partial parathyroidectomy.

But an analysis of the figures shows that a significant division may be made between all those that lived and those that died, on the basis of their blood calcium and phosphate levels at the onset of tetany (Chart 4). We may therefore turn our attention to this aspect of the situation and analyze the experimental findings from this point of view. The rabbits, regardless of the apparent state of their parathyroid glands, have been grouped accordingly in Table I.

In the first place, the control figures for both groups show less variation in the calcium levels than in those for phosphorus, before injection. Those that lived are not different in blood calcium from those that died, either individually or on the average. But in the group of eight survivors there are only three with a blood phosphorus, before injection, above 7 mg. per 100 cc. of serum, while among those that died nine of the eleven had high control levels, indicating, possibly, an inherent tendency to phosphorus retention.

As is shown by the available 2 hour determinations, the first peak of phosphorus in the blood probably occurred in most instances before the 4 hour specimen was taken. Of the nine rabbits examined at 2 hours, four survived and five died. Those that survived showed at the 4 hour period a considerable drop in blood phosphorus to a level which was maintained or further decreased at 5 to 7 hours. Some rabbits that died, on the other hand, had even higher levels at 4 hours than at 2, and the high concentrations of phosphorus in their blood at 5 to 7 hours are in marked contrast to the correspond-

TABLE I.

Lived

Rabbit No.	Condition	Serum Ca mg. per 100 cc.						Serum P mg. per 100 cc.					
		Control	Hrs. after injection					Control	Hrs. after injection				
			2	4	5–7	24	48		2	4	5–7	24	48
22	N	11.0	6.3	5.7	7.2	8.8	11.2	5.6	19.7	15.6	14.6	7.5	6.5
24	N	12.3	7.2	6.5	6.3	7.8	11.0	5.2	18.1	16.8	17.0	9.0	5.8
26	I	14.6	—	7.4	7.8	10.2	11.0	13.0	—	17.1	17.4	9.6	—
29	I	12.8	8.6	7.9	7.9	12.4	—	6.6	25.7	17.1	11.5	5.0	—
35	P P	12.8	—	7.4	—	6.4	5.8	15.3	—	20.6	—	18.0	7.4
34	P P	13.7	—	6.7	—	11.8	12.7	5.7	—	12.8	—	4.3	5.2
31	P P	12.9	—	7.3	—	10.8	11.4	5.8	—	15.1	—	4.7	4.9
37	I P P	10.8	7.5	6.7	8.1	9.2	—	9.5	29.2	23.8	15.7	6.0	—
Average.............		12.6		7.0	7.5	9.7	11.0	8.3		17.4	15.2	8.0	6.0

						Died							Death at
25	N	13.3	7.5	5.5	6.7			10.3	24.2	25.6	22.1		5 hrs., 18 min.
23	N	11.9	7.5	6.7	7.9			9.9	17.2	19.2	22.5		6.05
27	I	13.1	7.3	6.3	6.5			12.0	27.7	21.7	21.7		7–8 hrs.
28	I	12.0	8.6	8.2	8.1			6.2	24.0	18.4	24.8		First night
30	P P	13.0	—	7.1	7.3			6.8	—	14.8	33.0		7.05
32	P P	12.9	—	6.5	—			7.6	—	25.9	—		3.54
33	P P	13.5	8.8	7.5	8.4			15.0	24.2	23.1	38.7		6.28
40	I P P	12.8	—	6.5	7.3			7.6	—	21.8	28.4		7.18
39	I P P	11.8	—	6.7	7.7			8.8	—	21.4	30.0		6.53
36	I P P	12.9	—	7.5	—			8.2	—	15.4	—		5.27
38	I P P	13.1	—	6.3	9.6			8.8	—	19.2	24.0		5.30
Average............		12.8		6.8	7.7			9.2		20.6	27.2		

N = Normal.
I = Irradiated.
P P = Partially parathyroidectomized.
I P P = Irradiated and partially parathyroidectomized.

ing figures for the survivors.   The fate of these rabbits, therefore, depended not on the primary blood phosphorus level *attained shortly after injection*, but on the elimination or retention of this excess of

phosphorus in the course of the next few hours. Every rabbit (but one ?) that died had a blood phosphorus level above 21 mg. per 100 cc. serum, 4 to 7 hours after injection. Every rabbit that survived certainly or presumably had a blood phosphorus level below 21 mg. at the same period.

With reference to the occurrence of acute tetany, it is essential that the corresponding figures for blood calcium be taken into consideration. The 2 hour specimens showed a marked drop in blood calcium from the control figures, and this drop was even greater after 4 hours and often reached levels commonly associated with the signs of acute tetany, which all these animals displayed. But it is manifestly significant that differences in the blood calcium level do not distinguish the surviving rabbits from those that succumbed. In the rabbits that died, although the initial drop in blood calcium coincided with the great initial rise in blood phosphorus, the secondary rise in phosphorus that preceded death was in no observed instance accompanied by a further drop in calcium (11), so that just before death occurred the blood calcium in these rabbits was usually somewhat higher than it had been an hour or more previously. Low calcium alone cannot be held responsible for the death of these rabbits.

In the first paper of this series we developed the hypothesis that absolute figures for serum calcium or serum phosphorus are not so significant in experimental tetany as is the ratio between the two elements in the blood. Howland and Kramer (8, 12) have pointed out that in normal children this ratio is approximately 2:1, a proportion most favorable to bone formation, and that in rickets changes in the ratio occur, which may explain, in part, the failure of calcium phosphate deposition. Normally in rabbits also the ratio Ca/P is greater than 1. Among 103 rabbits the average ratio was 1.9:1. In these phosphate-injected animals, however, especially in the instances of phosphate retention in the blood, the ratio fell to a small fraction of its former value. These low ratios are obviously of more significance than are the individual calcium levels alone in interpreting the occurrence of severe and lethal tetany.

In considering the significance of these relative changes in Ca and P concentration, the fact that considerable amounts of the mono-

valent sodium ion were also injected probably must be taken into account.   One well established theory of hyperirritability and tetany is based on disturbances in balance between monovalent and divalent cations and anions in the blood.   In a very sane and illuminating review and discussion of this question Hastings and Murray (11) weigh the relative importance of monovalent cations and divalent anions as stimulants to nerves.   When disodium hydrogen phosphate is injected, a "double dose," so to speak, of nerve irritants is given, and it is hardly profitable to speculate as to which is the chief offender.

It is not our intention on the basis of these few experiments and those reported in the first paper of this series to undertake a critical discussion of current theories of the cause, or more probably causes, of experimental tetany.   This vexed and complicated question is not germane to the original object of this investigation.   That specific object was attained in the discovery that no significant differences were observable in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate.

SUMMARY.

No significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or disodium hydrogen phosphate.

Intravenous injections of calcium chloride caused only a transient rise in blood calcium, which returned to its former level within a few hours.   The upper level of blood calcium concentration is independent of parathyroid control.

Subcutaneous injections of disodium hydrogen phosphate caused, in all three groups of rabbits, a similar depression of the blood calcium level.   After large doses of the phosphate solution, all of the rabbits showed signs of acute tetany in which many of them died. Death or survival was not determined, apparently, by the absolute depression of blood calcium.   The rabbits that died were those, in all three groups, in which a phosphorus retention carried the initial rise in serum phosphorus to even higher levels in the hours preceding death, and so changed the normal calcium-phosphorus ratio to a small fraction of its former value.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

III. The Effects of Inanition.

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In order to test the functional significance of the parathyroid hypertrophy due to ultra-violet irradiation of normal rabbits (1) we have subjected irradiated rabbits to partial parathyroidectomy (2) and have compared the effects of injections of calcium chloride and of disodium hydrogen phosphate in irradiated, normal, and partially parathyroidectomized animals (3).

A third method of questioning the significance of this parathyroid hypertrophy was to observe the effects of complete inanition in irradiated, normal, and partially parathyroidectomized[1] rabbits to determine possible differences in the fasting metabolism of calcium and inorganic phosphorus with which the parathyroid glands, directly or indirectly, are so essentially concerned.

Much of the recent work on the physiological and therapeutic effects of ultra-violet light has been devoted to studies on various aspects of metabolic activity, especially with diets which are vitamine-poor, or incomplete in other respects. A fasting experiment with irradiated rabbits also promised opportunities for observation of certain effects of ultra-violet light on metabolism and further information in regard to the action of radiant energy on the endocrine glands.

Accordingly two experiments were undertaken, in which different groups of irradiated, normal, and partially parathyroidectomized rabbits were subjected to fasts of 5 and 11 days, respectively. The

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[1] All operations for removal of parathyroids were performed under full ether anesthesia.

5 day period proved too short to yield results of definite significance, and was complicated by the ingestion of unknown amounts of sawdust and feces from the individual cages, so it will not be reported in detail. In outline the experiment ran as follows:

Experiment 1.—This experiment was started with fifteen adult male rabbits in three groups. Five served as normal controls. Five had been irradiated for 44 days with a quartz mercury arc at 1 meter's distance with daily exposures of 10 minutes to 2 hours. The 2 hour exposure was continued during the fasting period. The other five rabbits had been partially parathyroidectomized with the removal of both external parathyroids and half the thyroid gland with its internal parathyroid 50 to 34 days previously. Except in one instance the remaining internal parathyroid gland in these rabbits was maintaining a calcium level of 12.0 to 13.4 mg. per 100 cc. serum. The exception, a rabbit with a control blood calcium of 9.3 mg. and inorganic phosphorus of 8.4 mg. per 100 cc. serum, died on the 5th day of the fast with a blood Ca of 5.7 mg. and P of 12.0 mg. per 100 cc. serum. The rabbits were kept in their regular sawdust-strewn individual cages and given 100 cc. of distilled water by stomach tube daily. Blood samples were taken from the ear before the fast, on the 2nd and 5th days, and 2 to 4 days after feeding had been resumed. The serum specimens were analyzed for Ca (4) and inorganic P (5).

One of the irradiated rabbits was killed by accident on the 5th day of the fast. Autopsy revealed a certain amount of hair, fecal matter, and sawdust in his stomach. Presumably the other rabbits also had ingested indefinite amounts of sawdust and feces, so that the results of this experiment are not open to a strict interpretation. But the average figures for each group show certain tendencies in calcium and phosphorus metabolism that lend emphasis to the results of the second experiment.

Eliminating from the averages for each group the figures obtained from the two rabbits that died and from two others, one normal and one partially parathyroidectomized, for which our records are incomplete, the results of this experiment are shown graphically in Chart 1.

A primary effect of fasting may be seen in the rapid fall in blood calcium that occurred in all of the rabbits. As might be expected, the total loss of calcium by the partially parathyroidectomized rabbits was somewhat greater than that in the other groups. In the first few days after feeding was resumed the blood calcium was restored almost as rapidly as it had been lost during inanition. *The more rapid rate of calcium accumulation in the irradiated and partially parathyroidec-*

tomized rabbits, as compared with the normals, may be due to a more active state of their parathyroid tissue.

A reciprocal relationship between calcium and inorganic phosphorus may be illustrated by the inverted curves for phosphorus in the irradiated and normal rabbits. Inorganic phosphorus in the blood serum was above normal in these animals during the fasting period and dropped again when the calcium supply was restored. The partially parathyroidectomized rabbits, on the other hand, lost both calcium and phosphorus from the blood;—except in the instance noted above (and not included in the average figures), in which a drop in calcium
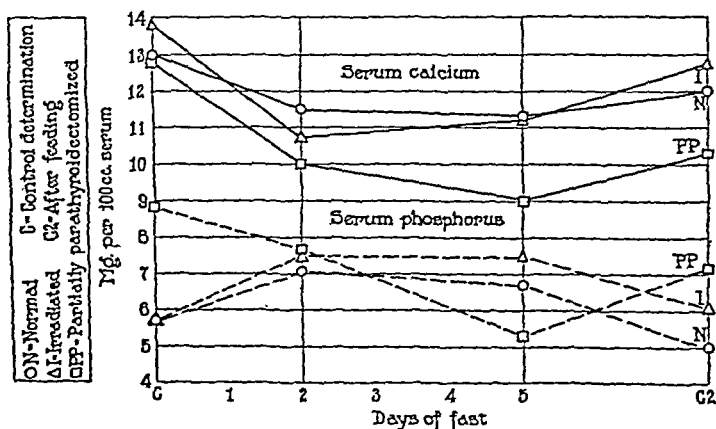

CHART 1. Experiment 1. Serum Ca and P during inanition in normal, irradiated, and partially parathyroidectomized rabbits.

to 5.7 mg. and a rise in phosphorus to 12.0 mg. per 100 cc. serum preceded the rabbit's death.

During this short fasting period none of the rabbits suffered an undue loss in body weight and the average losses for all three groups was less than 10 per cent.

After the final blood samples were obtained, the normal and the irradiated rabbits were killed and autopsied to determine the relative size of their external parathyroid glands. The two external glands of the irradiated rabbits averaged 8.1 mg. per kilo net body weight; those of the normal rabbits averaged 5.7 mg.

In the second experiment the individual metal cages were fitted with raised bottoms of wire mesh through which the urine and feces could fall freely, so that the rabbits ingested nothing but distilled water during the fast.

Experiment 2.—This experiment was performed with fifteen adult male rabbits in three groups. Five rabbits served as normal controls. Five rabbits with ears shaved and backs clipped had been irradiated 2 to 22 minutes daily (except Sundays) for a period of 20 days at 50 cm. distance from a 67 volt, 5.5 ampere quartz mercury arc. During the fast, daily exposures of 23 to 30 minutes were given. The other five rabbits had been deprived of their two external parathyroid glands 10 days previously. The three groups of rabbits were subjected to an 11 day fasting period.

The rabbits were each given 100 cc. of distilled water daily by stomach tube, and were bled from an ear vein for serum samples before the fast, on the 4th, 7th, and 11th days of inanition, and, after feeding had been resumed, on the 12th, 15th, and 18th days of the experiment. The serum samples were analyzed for calcium and inorganic phosphorus. The rabbits were weighed on alternate days.

Two rabbits, one irradiated and one partially parathyroidectomized, died on the 9th day of the fast, and since the figures for Ca and P obtained from them have been excluded from the averages in Chart 2 they must be dealt with separately. The irradiated rabbit started the fast with a serum Ca of 12.7 mg. and P of 8.8 mg. per 100 cc. serum. On the 7th day its serum Ca and P were 8.7 mg. and 10.7 mg. respectively. In 9 days of inanition this rabbit lost 52 per cent of its original body weight and its death is ascribed to causes associated with this great loss, rather than to tetany. The partially parathyroidectomized rabbit had not restored its blood calcium as promptly as is usual after such an operation (2) and started the fast with a serum calcium of 8.9 mg. and phosphorus of 8.8 mg. per 100 cc. serum. On the 4th day serum Ca was 7.2 mg. and on the 7th day 4.9 mg. The corresponding figures for P were 11.3 mg. and 12.7 mg. respectively. Although the death of this animal was not observed, such figures are typical of acute tetany.

Three of the four irradiated rabbits that survived the actual period of inanition died within the next 5 days. The normal and partially parathyroidectomized rabbits were allowed to survive in order to study the period of their recovery. 2 months later a second group of five normal rabbits was subjected to a similar fast and killed at its conclusion to provide a comparison at autopsy with the endocrine glands of the irradiated rabbits. In Chart 2 the figures for Ca and P in the serum of this second group of normal fasting rabbits have been averaged in the curves marked N2.

The figures for calcium and inorganic phosphorus (in mg. per 100 cc.

serum) in the blood of the rabbits which survived the actual period of inanition have been averaged for each group in Chart 2. With few exceptions these averages fairly represent the individual calcium findings. A bizarre and unexplained figure of 15.1 mg. for serum Ca in one of the irradiated rabbits, and a single high Ca of 12.45 mg. in one of the partially parathyroidectomized rabbits on the 4th day of the fast disturb the downward trend of the calcium averages, and have
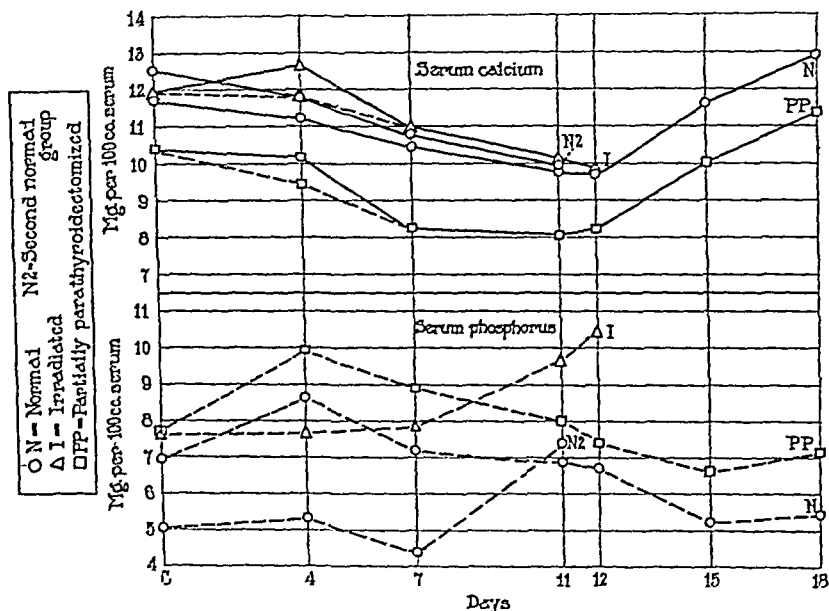

CHART 2. Experiment 2. Serum Ca and P during inanition and after feeding in normal, irradiated, and partially parathyroidectomized rabbits.

been omitted in the alternate averages for that day, also shown in the chart. The corresponding figures for phosphorus in these two animals were not different from those of other rabbits in the same groups.

As in the first experiment, inanition caused a progressive loss in serum calcium in all three groups of rabbits. A similar loss in serum phosphorus did not occur. We shall not attempt to interpret this tendency to phosphorus retention. Wide variations occurred in the figures for the individual rabbits and the averages merely cancel these

variations, so that no group stands out as manifestly different from the others in regard to phosphorus metabolism during the fast. Yet the fact that inanition does not regularly cause a progressive drop in the inorganic phosphorus of the blood serum is shown by the maintenance of the phosphorus levels in six of the seven groups in Experiments 1 and 2.

In regard to serum calcium and inorganic phosphorus metabolism, then, only one difference appears which can be interpreted as due directly to differences in parathyroid function among the four groups of rabbits. The lower level of serum calcium in the partially parathyroidectomized rabbits was maintained throughout both experiments. But the trend of the calcium level in this group was not different from that in the others.

Mention has been made of the fact that one irradiated rabbit died on the 9th day of the fast and that three of the four survivors died within 5 days after feeding was resumed. With the single exception of the partially parathyroidectomized rabbit that died on the 9th day, apparently of tetany, no similar fatalities occurred among the other groups. In general the 11 day period of inanition was well borne by all but the irradiated rabbits. The cause of the deaths in this group must be sought among the direct or indirect effects of the exposure to ultra-violet light. The dosage used, 5 to 30 minutes at 50 cm. distance, is not excessive and had been well borne by normal rabbits. Although the serum phosphorus was high in three of the four victims, serum calcium was not unduly low in the last determination made before death.

Aside from its hypertrophic effect on the parathyroid glands ultraviolet light has two possible effects on the animal which must be taken into account in this experiment. One is on general metabolism. The other is adrenal injury.

A comparison of the relative loss of weight by the three groups of irradiated, normal, and partially parathyroidectomized rabbits shows that from the first the irradiated animals lost weight more rapidly during inanition than did the other groups and toward the end of the fasting period this loss was exaggerated. Even during the first 4 or 5 days after feeding was resumed the weight of most of these rabbits remained stationary or declined further, and it was during this period

that three of the four surviving irradiated rabbits died, each at the nadir of his body weight curve. While the five normal rabbits lost an average of 31 per cent in body weight, and the four surviving partially parathyroidectomized rabbits lost 29 per cent, the five irradiated rabbits lost 52, 41, 39, 48, and 38 per cent of their control weights, respectively, and the first four of these rabbits died on the 9th, 12th, 13th, and 16th day of the experiment.

If loss in body weight be taken as an index of the metabolic rate in these rabbits, the effect of exposure to the quartz mercury lamp is at once apparent. In judging the effects of the exposure two factors have to be taken into account. One is the direct action of visible and ultra-violet light. The other is the handling and the movements of the rabbits due to the daily exposure. All of the rabbits were brought to the laboratory daily for water by stomach tube. In addition, the irradiated group was placed for about half an hour in a galvanized iron tub in which movement was not so restricted. In general they remained quiet, and we do not believe that this extra handling of the irradiated rabbits can account for their excessive losses of weight and for their deaths.

A review of the literature on the effects of visible and ultra-violet light on basal metabolism indicates that experimental conditions and methods have varied so widely that few useful comparisons can be made.

Thus Kestner, Peemöller, and Plaut (6), using a quartz mercury arc lamp in a study with the Benedict apparatus, produced a transient rise in metabolism in children and adults, but Hill and Campbell (7) call attention to the effects of cool air during exposure and consider that the rise in the metabolic rate caused by heliotherapy *per se* is relatively insignificant. Fries (8) found no appreciable difference in basal metabolism of children during treatment with a quartz mercury lamp compared with intervals without treatment, but thinks that the reaction of the individual must be taken into consideration. Recently Campbell (9) has reported a carefully planned and executed series of experiments with rats and mice in a specially adapted metabolism chamber. The oxygen consumption and the carbon dioxide output of four men were tested also, and the author concludes that irradiation with the whole or part of the spectrum of a quartz mercury vapor lamp has no effect on the metabolism of healthy men, rats, or mice. This is a sweeping statement in view of the fact that only the immediate or current effects of the radiation were investigated.

Northrop (10) in a precise experiment with aseptic cultures of *Drosophila*, grown for 200 generations in the dark, found that the light of a tungsten filament

lamp, through water, had marked effects on growth and duration of life. With intensities greater than 1000 meter candles the duration of the imago stage was rapidly shortened and the duration of life of the imago could be predicted by assuming that the light ages the flies independently of the normal rate. The light effect was proportional to its intensity.

In the absence of observations on basal metabolism in our groups of fasting rabbits, we can only call attention to the excessive loss of weight in the irradiated rabbits, and suggest its possible relation to an increased metabolic rate resulting in the more rapid consumption of the animal's own tissues.   A definite effect of fasting and the exposures to radiant energy was found at autopsy in the irradiated victims of the fast.

From time to time we have observed gross hemorrhages in the suprarenal glands of irradiated rabbits (1).   The point of origin of these hemorrhages has usually been the medullary tissues, but they have often extended into the cortex and even to the capsule of the affected gland.   The frequency of such a lesion after irradiation, even when only moderate erythema doses have been given, has raised in our minds the question of a specific secondary action of ultra-violet energy on these essential organs.   The effects of such an injury might be reflected clinically in blood pressure changes, which were not studied in these fasting rabbits.   Under other conditions, light, especially ultra-violet light, has often been observed to lower blood pressure (11) and the signs of collapse that sometimes follow dangerous degrees of sunburn are in certain respects similar to the signs of suprarenal insufficiency (12).

According to Jackson (13), and other authors whom he quotes, inanition and various types of malnutrition often cause suprarenal congestion and hemorrhage.   In fasting the suprarenals do not show a proportionate loss in weight, sometimes being relatively twice as large as is normal. Congestion, and sometimes hemorrhage, is characteristic in both cortex and medulla and may account for the increased weight in spite of atrophy of the parenchyma.   Congestion and hemorrhage, gross or capillary, have been reported also in athrepsia, scurvy, and pellagra, so it appears that the suprarenal glands often suffer heavily in states of malnutrition.

With two causes of suprarenal damage operating in conjunction, it

is hardly surprising that three of the four irradiated rabbits examined at autopsy showed scars and medullary hemorrhage in one or both of these essential glands.   One gland of the fourth rabbit was lost, and its condition cannot be reported.   The other gland appeared to be normal.   None of the lesions were so recent as to suggest that they were agonal, or the immediate cause of death, but suprarenal insufficiency may have contributed to the high mortality among the irradiated rabbits.

TABLE I.

Glands	Gland weights (in mg. per kg. net body weight)				Change in gland/body weight ratio, effect of					
	Normal		Irradiated		Irradiation		Fasting		Both	Fasting
					I	II	III	IV	V	VI
	Controls	Fasted	Controls	Fasted	IC/NC	IF/NF	NF/NC	IF/IC	IF/NC	Jackson
Pineal	6.7	11.3	10.2	14.1	+	+	+	+	+	±
Hypophysis	14.8	18.2	16.9	29.6	+	+	+	+	+	+
External parathyroids	6.1	5.5	9.2	7.0	+	+	−	−	+	−
Thyroid	112.8	116.4	92.2	156.2	−	+	±	+	+	+
Thymus	1151	390.	1111.	402.	±	±	−	−	−	−
Suprarenals	195.0	279.7	212.5	374.6	+	+	+	+	+	+
Testicles	2828.	3122.	2670.	3066.	±	±	+	+	+	−

N = Normal. I = Irradiated. C = Control. F = Fasted.

+ = Gland spared. Weight of gland increased relative to body weight.

− = Gland consumed. Weight of gland decreased relative to body weight.

At autopsy other endocrine glands of the fasted, irradiated rabbits, and of the fasted, normal rabbits of Group N2, were weighed and their weights compared with figures obtained for other irradiated and normal rabbits at a corresponding season of the year.   These figures and a crude analysis of them have been collected in Table I, which may require some explanation.

The figures given represent gland weights, in mg. per kilo of net body weight, after the removal of the gastrointestinal tract and its contents, and the expression of bladder urine.   For the normal control figures we are indebted to Dr. Louise Pearce.   In the second half

of the table an attempt has been made to indicate whether fasting spares these essential glands, or exhausts and consumes them more rapidly than other body tissues. The effect of irradiation alone is seen in the gland weight ratios of irradiated and non-irradiated rabbits in both the control and the fasting groups. An idea of the effect of fasting alone is gained by comparing fasted with control rabbits in the normal and irradiated groups. A summation of the effects of irradiation and fasting is found in a comparison of the figures for the irradiated fasted rabbits with the normal controls. Considering the small number of fasted rabbits available for study, the results of the analysis are in good accord. The effects of irradiation alone, Columns I and II, check in most instances both for normal and for fasted rabbits and in the control, irradiated, rabbits the same effects of irradiation are seen as have already been reported (1): pineal, hypophysis, external parathyroid, and suprarenal glands are relatively heavier than normal, the thyroid gland has lost weight, and the thymus and testicles show little change. Similarly the effects of inanition on these endocrine glands may be seen in Columns III and IV. Pineal, hypophysis, thyroid, suprarenals, and testicles are spared in both groups of rabbits. The external parathyroids, and especially the thymus are consumed relatively faster than the body as a whole. Finally, in the single instance in which the effects of fasting and of irradiation on gland weight seem to be opposite, *i.e.* the effect on the external parathyroid glands, the light treatment has more than offset the effects of fasting, and the parathyroids in the irradiated fasted rabbits are larger than is normal. But in spite of the relatively large size of the external parathyroid glands in the irradiated rabbits, their curves for serum calcium during the fasting period in both experiments are essentially similar to those for the normal groups (see Charts 1 and 2).

In most instances these tendencies to the protection or exhaustion of the endocrine tissues in our fasted rabbits correspond with the effects of inanition in man and other animals, reviewed and summarized by Jackson (13). His general conclusions in regard to each gland are indicated in Column VI in the table, and may be noted briefly as follows:

Pineal Gland.—No appreciable change relative to body weight. No conclusions drawn.

Hypophysis.—Loss of weight relatively less than that of the body as a whole.

Parathyroid Glands.—Usually found to be atrophic. In rats Jackson found them reduced in weight nearly in proportion to the body loss. They tend to be relatively decreased in weight in adults, with a variable degree of atrophy and an increase in fibrous stroma.

Thyroid Gland.—The loss of weight is variable, usually relatively less than that of the body as a whole.

Thymus.—The extreme loss of weight of this gland is rivaled only by that of adipose tissue. The loss is relatively far greater than that of the body and usually reaches 75 per cent before death from acute inanition.

Suprarenals.—These glands are usually increased in weight but part of this increase may be due to hemorrhage.

Testicles.—The loss in weight is relatively greater than that of the body as a whole.

Thus the only result of our observations that is at variance with Jackson's conclusions is in the loss of weight of the testicles relative to general body weight loss. But these glands normally vary widely in absolute and relative weight, and we lay no stress upon the aberrant results of our single experiment.

SUMMARY AND CONCLUSIONS.

The experiments described in the three papers of this series were undertaken to determine if possible the significance of the parathyroid hypertrophy that has repeatedly been found in rabbits after exposure to ultra-violet light. The fact that the hypertrophy was not accompanied by a corresponding increase in blood calcium led us to infer that the upper level of blood calcium concentration is governed by factors independent of parathyroid control, and that the gland hypertrophy under the influence of ultra-violet light might represent a potential functional capacity, or increased factor of safety, to protect the calcium level under conditions of stress or emergency. Partial parathyroidectomy in irradiated rabbits, compared with the same operation in normal controls, resulted in a similar immediate drop in blood calcium; but a more rapid restoration of the calcium level in the irradiated rabbits indicated a more prompt and active response to the emergency on the part of the remaining glands. In the second group

of experiments no significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate. All three groups of rabbits rapidly eliminated the excess calcium from the blood. Their reactions to large doses of sodium phosphate, given subcutaneously, appeared to be related to phosphate elimination or retention in the individual animals. Those in each group which had phosphate retention in the blood died in acute tetany.

So also in the fasting experiments, reported in this paper, no distinctions could be drawn between normal, irradiated. and partially parathyroidectomized rabbits on the basis of their calcium and phosphorus metabolism. Blood calcium was reduced in a similar manner in all three groups of rabbits, and wide individual variations in serum phosphorus levels preclude significant deductions.

But the irradiated rabbits suffered disproportionate losses of weight during the fasting period and four of five of them died during the course of the experiment. The deaths of these rabbits are attributed to this more rapid consumption of their own tissues and possibly also to suprarenal injury caused both by fasting and by exposure to ultraviolet light.

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STUDIES ON BACTERIAL ENZYMES.

I. MENINGOCOCCUS MALTASE.

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INTRODUCTION.

The fermentation of maltose is one of the most important characters employed in the differentiation of meningococci from some of the saprophytic cocci found in the respiratory tract. It is of importance, therefore, to determine whether maltose hydrolysis can be effected by enzymes liberated from the bacterial cells. In the present paper this question has been answered by an investigation of the maltose-splitting capacity of sterile filtered solutions of the intracellular substances of meningococci.

Measurement of the action of maltase involves an estimation of glucose in the presence of maltose. Since both are reducing sugars, glucose in such mixtures cannot be estimated by the usual, simple or direct copper reduction methods. Polarimetric determination of glucose requires special apparatus not always at hand in the bacteriological laboratory. Barfoed's method is applicable to the detection of glucose in the presence of maltose, but requires rigid controls especially when the amount of maltose greatly exceeds the glucose. Preliminary tests by this method on mixtures of the bacterial enzyme and maltose did not prove satisfactory. Precise readings were difficult to make because of the voluminous precipitation of the phosphate in the test solutions by the acetic acid in Barfoed's reagent.

Since only qualitative estimations were desired, a biological method was employed, which is based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

EXPERIMENTAL.

Methods.

Preparation of Meningococcus Enzyme Solutions.—The enzyme solution was prepared from a strain of normal meningococci (Type II Gordon) furnished by Dr. Elliott S. Robinson of the Massachusetts Antitoxin and Vaccine Laboratory.

Meat infusion agar enriched by the addition of Avery's (1) yeast extract was inoculated heavily with young meningococci suspended in infusion broth. Luxuriant growth was always obtained within 12 to 18 hours; the meningococcus cells were then scraped off the surface of the agar and suspended in sterile 0.1 M phosphate (pH 7.5). Since the medium in which the bacteria were suspended included the broth introduced with the original inoculum, the final solution in which the bacteria were extracted contained about an equal volume of infusion broth and phosphate solution. The meningococcus suspension was placed in long narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus extracts (2).

The suspension of disintegrated bacteria was centrifuged at high speed for 1 hour; the supernatant was then pipetted off from the bacterial residue. To eliminate any possibility of the presence of intact meningococcus cells in the enzyme solutions, the supernatant was finally filtered through a Berkefeld candle.

Preparation of Maltose Solutions.—To avoid the hydrolysis which frequently accompanies the sterilization of dilute solutions of maltose, the sugar was heated in concentrated solutions. 30 per cent solutions of maltose were boiled for 15 minutes and then added aseptically to sterile 0.1 M phosphate (pH 6.9) in amount sufficient to give a final concentration of 3.0 per cent maltose. The final maltose solution was distributed into sterile Pyrex tubes.

The Detection of Glucose by Bacterial Acid Fermentation.—The procedure used to detect glucose by bacterial acid fermentation consisted in the following steps: (1) the preparation of hydrolysis mixtures (maltose plus meningococcus enzymes); (2) the incubation of the hydrolysis mixture to allow time for the action of the enzyme upon maltose; (3) the addition of certain glucose-fermenting bacteria to samples of the hydrolysis mixtures and measurement of the acid produced by the bacterial fermentation of glucose.

1. "Hydrolysis Mixtures."—The hydrolysis mixtures consisted of maltose solution plus unheated, active meningococcus enzyme solution and control mixtures of maltose solution plus heat-inactivated enzyme solution. These mixtures were incubated for 48 or 72 hours to allow time for the enzyme action.

2. "Fermentation Mixtures." (a) *Bacterial Fermenting Agents Employed.*—The bacteria used to detect the presence of glucose consisted of a strain of Shiga dysentery bacillus and an atypical strain of colon bacillus. (The strain of Shiga dysentery bacillus possessed the usual fermentation characteristics of these bacteria. The atypical strain of colon bacillus was isolated from stools by Mr. William L. Fleming. This strain, at least when first isolated, fails to ferment maltose al-

though actively attacking both glucose and lactose with prompt acid and gas formation, which is a comparatively rare combination of fermenting properties among the known intestinal bacteria.) Ideal fermenting agents for the detection of glucose in mixtures of maltose and glucose should possess the property of fermenting the hexose with acid production but should be without action upon maltose. Although it was found that concentrated suspensions of the strains used in our experiments do form small amounts of acid in maltose solutions, it simplifies the description of the method to define them as "non-maltose-fermenting bacteria." Any error from the slight fermentation of maltose by the "non-maltose-fermenting" bacteria was eliminated by the rigid controls described below.

The bacteria actually used as fermenting agents were taken from young agar cultures and were suspended in equal parts of meat extract broth and salt solution.

(b) *Preparation of the "Fermentation Mixtures."*—The "fermentation mixtures" were prepared as follows: An aliquot of each hydrolysis mixture, usually 0.5 cc. or 0.7 cc., was put into a series of small sterile Pyrex test-tubes. Then an equal volume of a suspension of the bacterial fermenting agent was added to each of the samples under test for the presence of glucose.

Although the reaction of the hydrolysis mixture was pH 6.9, it was our practice to adjust the initial reaction of the fermentation mixture to approximately pH 7.5. Not only is this pH nearer the optimum for acid fermentation, but with the initial reaction at pH 7.5 the production of small amounts of acid causes larger changes in pH than at pH 6.9 where the "buffer value" of the phosphate is at its maximum. A further advantage of having the more alkaline initial reaction is that smaller decreases in pH can be accurately estimated between pH 7.5 and pH 6.9 by the use of phenol red (3), than can be detected with brom-cresol purple in a zone slightly below pH 6.9. The reaction of the bacterial suspensions, therefore, was adjusted to approximately pH 8.5 which trial tests showed was sufficiently alkaline to yield a pH of 7.5 in a mixture of equal parts of the bacterial suspension and pH 6.9 (0.1 M) phosphate.

(c) *Incubation of "Fermentation Mixture" for Acid Fermentation of the Glucose.*—The "fermentation mixtures" (test solution plus glucose-fermenting bacteria) were shaken and then incubated in the water bath at 38°C. for 45 minutes. The factors to be considered in choice of the length of the incubation period will be considered below (Fig. 1).

(d) *Estimation of Acid Produced in the "Fermentation Mixture."*—0.5 cc. portions of properly diluted solutions of phenol red or of brom-cresol purple were placed in a series of small selected tubes of equal bore. Colorimetric standards were prepared by adding 0.5 cc. of buffer mixture to the appropriate indicators. 0.5 cc. portions of the centrifuged supernatants of the fermentation mixtures were then added to tubes containing the indicators. These supernatants were themselves practically water-clear and were diluted with an equal volume of the indicator solution so that accurate readings could be obtained by direct comparison with the buffer standards.

Controls on Acid Production in the "Fermentation Mixtures."—The following controls were used to limit the changes in reaction of the fermentation mixture to

the acid formed by the fermentation of glucose previously hydrolyzed by the active meningococcus enzymes.

1. Errors due to any hydrolysis of the maltose by some other agency than the active meningococcus enzyme were eliminated by a control series of heat-inactivated enzyme plus maltose. This series was incubated under the same conditions as the corresponding mixtures of active enzyme plus maltose.

2. In some of the experiments, slight differences existed in the initial pH of the different mixtures of enzyme plus maltose. This was controlled by preparing a duplicate series of "fermentation mixtures" in which heat-killed bacteria were used instead of active bacteria.

Slow Fermentation of Maltose by Shiga Dysentery Bacilli.

In the preceding description of the detection of glucose in the presence of maltose by acid fermentation, it was convenient to speak of the Shiga dysentery and atypical colon bacilli used for fermenting agents as "non-maltose-fermenting bacteria." Cultures of these strains made in maltose broth with the usual sizes of inoculum showed no reddening of Andrade indicator after 48 hours incubation. Although this would be sufficient to classify the strains as "non-maltose fermenters" in a routine study of intestinal bacilli, a gradual fermentation of maltose even under the routine conditions was indicated by a faint reddening of the indicator after 1 or 2 weeks storage of cultures at room temperature. In such cases, however, the question of maltose-fermenting capacity is complicated to some extent by the possibility of a gradual hydrolysis of maltose in dilute (1 per cent) solutions.

The question of the extent of maltose fermentation by Shiga dysentery bacilli assumed importance in determining the period of time that should be allowed for the incubation of the final fermentation mixtures of maltose and glucose with the fermenting agents. The mixture, obviously, should be incubated long enough to insure the complete fermentation of the glucose, but if maltose is slowly fermented the incubation period should not be extended too long or the acid formed from the larger amount of maltose will obscure the acid formation from the hexose. Even a slight capacity to produce acid from maltose, which, with the fewer number of cells in a routine broth culture, would result in only traces of acid, may result in a

very significant amount of acid in tests with the concentrated suspensions of bacilli used in our experiments.

Preliminary tests were made to determine the most desirable period of incubation to allow the maximum acid formation from glucose with the minimum acid production from the maltose. A typical experiment is outlined below.
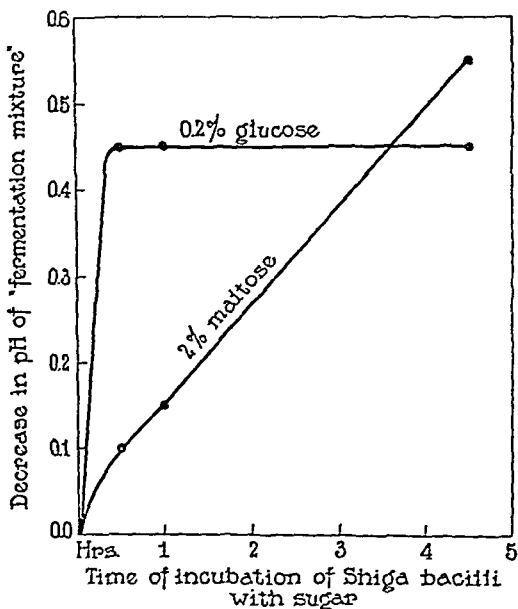

FIG. 1. "Slow fermentation" of maltose by Shiga dysentery bacilli. Relative rates of acid production by action of concentrated suspensions of the bacilli upon glucose and maltose.

Three tubes were prepared: (1) 5 cc. pH 7.6 phosphate solution plus 1.0 cc. sterile H_2O; (2) 5 cc. phosphate plus 1.0 cc. 20 per cent maltose solution; (3) 5 cc. phosphate plus 1.0 cc. 2 per cent glucose. To each of these tubes 3 cc. of concentrated suspension of Shiga dysentery bacilli were added. The final mixtures, therefore, represented "fermentation mixtures" of the same initial pH and the same buffer value containing respectively: (1) no sugar, (2) 2 per cent maltose, (3) 0.2 per cent glucose.

The mixtures were shaken and placed in the water bath. Samples were removed and pH measurements made after centrifugation at the end of 30 minutes, 1 hour, and 4 hours. The results are given in Fig. 1.

Fig. 1 illustrates the differences in rates of fermentation of glucose and maltose by our strain of Shiga bacilli. It is seen that glucose is fermented extremely rapidly, since the maximum acidity was reached within 30 minutes. Maltose, on the other hand, was fermented slowly as only a slight increase in acidity (0.1 pH) was evident at the time the maximum amount acid had been formed from the glucose. The continued, although gradual, increase in acidity in the 2 per cent maltose mixture (Fig. 1) emphasizes the necessity of avoiding prolonged incubation periods since it is obvious that the acid formed from traces of glucose would in time be obscured if an excess of maltose is present in the system.

From the relations revealed in this and other preliminary experiments it was concluded to adopt 45 minutes as the period of incubation of the test "fermentation mixtures." This period was greater than that required for the complete fermentation of the glucose with concentrated suspensions of the fermenting agents. In all cases, therefore, it was necessary to control the slight amount of acid produced from the maltose by the methods previously described.

All that is desired here is to establish the conditions for detection of glucose in the presence of maltose. Whether the "slow" fermentation of maltose is due to the presence of a small proportion of maltose-fermenting variants, or to a very slight maltose-fermenting capacity inherent in all dysentery bacilli, is of relatively little importance. A slight acid formation by Shiga bacilli in maltose solutions has been ascribed by some investigators to traces of glucose resulting from the heating of maltose during sterilization, or from a gradual hydrolysis in media which have been stored for a long time before use. Fig. 1, however, shows clearly that this explanation does not hold for the gradual fermentation of maltose by our strain. The differences in the form of the curves of acid production in the glucose and maltose can be due only to differences in rates of fermentation of the two sugars, since the curves would be similar in nature if the acid production in the "maltose solution" were due to traces of glucose. The above results, therefore, are also of importance as evidence that the production of small amounts of acid in maltose solutions by at least some strains of Shiga dysentery bacilli is a definite property which certainly cannot be explained by the presence of traces of glucose in the sterilized maltose.

Activity of Meningococcus Maltase and the Influence of Concentration of the Enzyme Solution upon Glucose Formation.

In the following experiment, the maltose-splitting activity of sterile, filtered solutions of meningococci is tested by the biological method just described. The relation of the degree of maltose hy-

TABLE I.

Influence of Concentration of Enzyme upon Glucose Formation by Meningococcus Maltase.

Hydrolysis mixture Amount of enzyme solution added to maltose solution		Hydrolysis mixture treated with the Fleming strain of colon bacilli	Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to fermentation of the glucose previously formed by meningococcus maltase enzyme (determined by fermentation with)	
		Unheated bacilli	Unheated bacilli	Fleming strain of colon bacilli	Shiga dysentery bacilli
cc.		*pH*	*pH*	*pH*	*pH*
2.0	Active enzyme	6.3	6.2	0.9	1.0
	Heat-inactivated enzyme	7.2	7.2		
1.0	Active enzyme	6.4	6.3	0.7	0.8
	Heat-inactivated enzyme	7.1	7.1		
0.5	Active enzyme	6.8	6.8	0.3	0.3
	Heat-inactivated enzyme	7.1	7.1		
0.2	Active enzyme	7.0	7.0	0.1	0.1
	Heat-inactivated enzyme	7.1	7.1		
0.1	Active enzyme	7.1	7.1	0 0	0.0
	Heat inactivated enzyme	7.1	7.1		
0.0	(Control)	7.1	7.1	0.0	0.0

drolysis to the concentration of the bacterial enzyme is demonstrated by tests made upon mixtures containing different concentrations of the meningococcus cell solution. The protocol of the experiment is summarized in Table I.

The results presented in Table I demonstrate that meningococci possess an enzyme which hydrolyzes maltose. The glucose formed

by the splitting of the maltose was detected by the formation of acid by glucose-fermenting bacteria. Too many factors are involved for one to attempt to show a precise quantitative relation between the different pH increments and the actual amount of glucose formed. However, the fact that the largest amounts of acid were formed in the mixtures in which the enzyme concentration was greatest, indicates that there existed a direct relation between the concentration of the meningococcus maltase and glucose formation.

TABLE II.

Comparison of Action of Meningococcus Enzyme Solution upon Sucrose, Raffinose, and Maltose as Indicated by Changes in Reactions.

Hydrolysis mixture		Change in pH due to fermentation of the hexose previously formed by meningococcus enzyme
		pH
Maltose	Active meningococcus enzyme solution	0.9
	Heat-inactivated meningococcus enzyme solution	0
Sucrose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0
Raffinose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0

Action of Meningococcus Enzyme Solution upon Sucrose and Raffinose.

The preceding experiment has demonstrated that meningococci possess a maltase enzyme. Since the method of demonstration of the maltase was indirect, it seemed important to apply the same biological method to solutions of sucrose and raffinose, neither of which is fermented by meningococci.

Solutions of sucrose and raffinose were prepared like the maltose solution. 2 cc. of active enzyme were added to 3 cc. of each of the sugar solutions; a second series was prepared with the same amount of heat-inactivated enzyme. The hydrolysis mixtures were incubated at 37°C. for 72 hours. The protocol of the experiment is summarized in Table II.

From Table II, it is seen that no acid is formed by hexose-fermenting Shiga bacilli when added to previously incubated mixtures of meningococcus enzymes and sucrose or raffinose solutions. This fact proves that the meningococcus cell solution hydrolyzes neither sucrose nor raffinose. Since it is commonly assumed that the inability of bacteria to ferment certain disaccharides or trisaccharides is due to their inability to hydrolyze the sugar, the above demon-

TABLE III.

Heat Lability of Meningococcus Maltase.

Hydrolysis mixture — Maltose solution plus meningococcus enzyme solution		Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to the fermentation of the glucose previously formed by action of the meningococcus maltase	
Treatment of enzyme solution	Amount of enzyme solution		2.0 cc. enzyme	1.0 cc. enzyme
	cc.	*pH*	*pH*	*pH*
Unheated	2.0	6.2		
	1.0	6.4	0.9	0.6
Heated 10 min. at 45°C.	2.0	6.6		
	1.0	6.7	0.5	0.3
Heated 10 min. at 50°C.	2.0	6.8		
	1.0	6.9	0.3	0.1
Heated 10 min. at 55°C.	2.0	7.1		
	1.0	7.0	0.0	0.0
Heated 10 min. at 60°C., 65°C.; 70°C.; 80°C.; 100°C.	2.0	7.1		
	1.0	7.0	0.0	0.0

strated absence of sucrase and raffinase is to be expected. However, the fact that significant increases in acidity occur only in the maltose solution treated with enzyme is important as evidence of the validity of the described biological method as a means of demonstrating a bacterial maltase enzyme.

Heat Lability of Meningococcus Maltase.

The following experiment was made to determine the relative degree of heat lability of meningococcus maltase.

Tubes of enzyme solution, sealed with vaseline to prevent oxidation at the higher temperatures, were immersed in a water bath that was constantly stirred, and heated for 10 minutes at the following temperatures: 45°, 50°, 55°, 60°, 65°, 70°, 80°, and 100°C. Samples of 2.0 cc. and 1.0 cc. of each of the heated enzyme solutions were added to separate tubes containing 3 cc. of 3 per cent maltose. The hydrolysis mixtures were incubated for 72 hours at 37°C. and were then tested for glucose by the fermentation method used in the preceding experiments. The protocol is summarized in Table III.

It is evident from the results of this experiment (Table III) that meningococcus maltase is entirely inactivated by 10 minutes exposure to 55°C. Since Table I has shown that the concentration of active enzyme is related to the amount of acid produced by the glucose-fermenting bacteria, differences in the final acidity offer a rough index of the relative amount of enzyme remaining active after the various heatings. When analyzed from this standpoint, the above results show that the enzyme is destroyed to some extent when heated to temperatures lower than 55°C. A significant loss in activity occurs during 10 minutes exposure at 45°C., and only a small proportion of the maltase remains active after 10 minutes at 50°C. Thus, this enzyme, like the carbohydrate-hydrolyzing enzymes of pneumococci (4) is an extremely heat-labile substance.

Endocellular Nature of Meningococcus Maltase.

The method of preparation of the meningococcus derivatives which we have studied indicates that the maltase is an endocellular substance freed when the bacterial cell is disrupted. However, to obtain actual proof of its endocellular nature the following comparison was made of the maltase activity of the supernatant fluid of a broth culture with the activity of a solution prepared from the centrifuged cells of the same culture.

1 liter of a 24 hour yeast extract broth culture of meningococci was centrifuged, and the supernatant removed from the bacterial sediment.

The meningococcus cells were collected and subjected to the freezing and thawing process. Both the supernatant fluid and the cell solution were finally filtered through a Berkefeld filter. The comparison of the maltase activity of the two preparations was made by the procedure used in the preceding experiments.

The results are summarized in Table IV.

The results of this experiment (Table IV) prove that meningococcus maltase is an endocellular substance, since maltase activity is exhibited by sterile solutions prepared from the bacterial cells and not by the supernatant or filtrate of the unautolyzed broth

TABLE IV.

Endocellular Nature of Meningococcus Maltase.

Hydrolysis test mixture	Change in pH due to fermentation of glucose formed by meningococcus maltase	Maltase activity
Maltose plus filtered solution of meningococcus cells	0.8	+
Maltose plus filtrate of broth culture of meningococcus	0.0	—

culture. Needless to say, the endocellular nature of the enzyme does not preclude the possibility of a demonstration of maltase activity by filtrates of broth cultures in which considerable cell autolysis has occurred (5).

DISCUSSION.

Studies on the enzymes of pathogenic bacteria are of more than academic interest. The life and growth of the bacteria, either in artificial culture or in the infected host, are dependent upon their metabolic functions, most of which in turn depend upon the action of enzymes. Knowledge concerning the enzymes leads to a better understanding of the mechanism of the biochemical processes exhibited in the test-tube and of the changes accompanying the growth of the bacteria in the body and the factors concerned in the satisfaction of their nutritional needs. The endocellular nature of many of the enzymes introduces a further point of interest since these enzymes must be considered as actual constituents of the bacterial cell which, in the case of pathogenic microorganisms, would be liberated into the tissues of the host if lysis of the bacteria occurs at any stage of the infection.

The results of the experiments presented in this paper demonstrate the maltose-hydrolyzing activity of filtered solutions of the intracel-

lular substances of meningococci. Hence, the splitting of maltose, the first step in the acid fermentation of this sugar by living meningococci, can be referred to a maltase enzyme the activity of which is independent of the presence of intact bacterial cells. The amount of maltose hydrolysis effected by the meningococcus maltase bears a direct relationship to the concentration of the bacterial enzyme. The maltase of meningococci, like the carbohydrate-splitting enzymes of pneumococci, is an extremely heat-labile substance. It is inactivated by prolonged exposure to 45°C., and is entirely destroyed by a short exposure to 55°C.

SUMMARY.

Meningococci possess an endocellular maltase enzyme. The splitting of maltose by this enzyme has been demonstrated by a biological method based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

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STUDIES ON BACTERIAL ENZYMES.

II. MENINGOCOCCUS PEPTONASE.

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INTRODUCTION.

The present paper reports a study of the peptone-hydrolyzing enzyme of Meningococcus. The activity of this enzyme has been studied with sterile solutions of the bacterial substances, not only devoid of living bacteria but freed of bacterial cells by Berkefeld filtration.

The maltase of Meningococcus has been demonstrated in the preceding paper (1). The fragility of meningococci and the readiness with which they undergo dissolution, facilitate the preparation of sterile solutions of their intracellular substances. A search of the literature, however, has revealed little work on the enzymes of Meningococcus. Flexner (2) in his early studies on Meningococcus demonstrated experimentally the enzymatic nature of the processes involved in the dissolution of the bacteria which occurs in laboratory cultures. He recognized the intracellular nature of the autolytic enzyme and studied the conditions which influence its operation. Since that time, there have been numbers of incidental reports on the autolysis of meningococci without, however, evidence referring the process to a bacteriolytic enzyme. Sturges and Rettger (3) studied the autolysis of suspensions of meningococci and observed that increases in amino nitrogen accompanied the morphological disintegration of the bacterial cells. Their paper is concerned, however, with autolysis of bacterial suspensions and does not furnish a demonstration of an active enzyme operative in solutions filtered free from bacteria.

EXPERIMENTAL.

Methods.

Preparation of Enzyme Solutions.—The earliest reports on bacterial enzymes were concerned with the so called "exocellular" enzymes which are liberated or

secreted into the culture medium during the growth of the bacteria. It is now recognized, however, that many of the enzymes of bacteria are more intimately bound to the microbial cell and are set free into the surrounding medium only upon its disintegration and the experimental demonstration of these enzymes depends upon their liberation either by imposed physical or chemical conditions or by autolytic processes. Since the enzymes are labile substances, it is important to prevent their deterioration or inactivation during the processes mentioned. Studies (3) on the oxidation of pneumococcus enzymes present many examples of the importance of protection of enzyme solutions from air during their preparation.

The meningococcus enzyme solutions used in this study were derived from a strain of normal meningococci and were prepared by the method described in the preceding paper (1).

Substrates.—2 per cent solutions of Bacto-peptone or proteose peptone were prepared in 0.1 M phosphate solution (pH 7.6). The peptone solutions were sterilized in the autoclave.

TABLE I.

Hydrolysis of Peptone by Sterile Solutions of Meningococcus.

NH$_2$-N per 100 cc. of hydrolysis mixture		
Heat-inactivated enzyme	Active enzyme	Increase due to action of enzyme
mg.	*mg.*	*mg.*
34.0	46.5	12.5

Amino Nitrogen Determinations.—The increases in amino nitrogen were determined by direct analysis of the enzyme-substrate mixtures by Van Slyke's nitrous acid method.

Sterility Control.—No antiseptics were added to any of the hydrolysis mixtures. Sterility was controlled by cultural methods.

Action of Enzymes of Meningococcus upon Peptone.

The first step in the investigation consisted in tests of the action of the sterile solutions of meningococci upon peptone. It is unnecessary to describe these experiments since the protocol given in Table I is sufficient evidence of the presence of an active enzyme which hydrolyzes peptone in the complete absence of living or formed bacterial cells.

In the experiment reported in Table I, 0.5 cc. of the active enzyme solution was added to 5.0 cc. of sterile peptone solution; an equal amount of enzyme previously inactivated by heat was added to another tube of the peptone substrate to serve as control of the active enzyme. After 36 hours incubation, amino nitrogen analyses were made to determine the extent of peptone hydrolysis. The protocol is given in Table I.

The protocol given in Table I is evidence of the active enzyme of Meningococcus which retains its ability to hydrolyze peptone in the complete absence of living or formed bacterial cells.

Heat Lability of the Peptonase Enzyme of Meningococcus.

All of the usual bacterial enzymes are sensitive to heat. The following experiment investigated the degree of thermolability of the peptonase enzyme of Meningococcus.
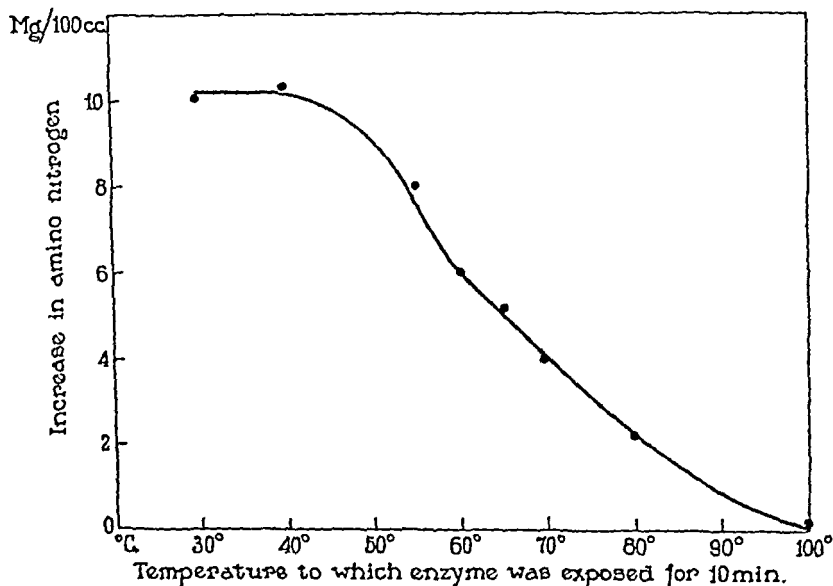

FIG. 1. Heat lability of meningococcus peptonase.

Portions of the enzyme solution (pH 7.4) were heated for 10 minutes at 40°, 55°, 65°, 80°, and 100°C. Equal amounts of the heated enzyme were added to a series of tubes containing 5 cc. of sterile peptone solution. A control tube of peptone to which unheated enzyme was added was used to determine the initial activity of the enzyme. The results of the experiment are presented in Fig. 1.

As shown in Fig. 1, the endocellular peptonase enzyme of Meningococcus is a heat-labile substance. The rate of its inactivation in-

creases with increase in temperature, and it is entirely destroyed by exposure to 100°C. for 10 minutes.

Intracellular Nature of Peptone-Splitting Enzymes of Meningococci.

The fact that the active enzymes demonstrated by the experiment yielding the figures given in Table I were prepared by disruption of the bacteria, in itself indicates that the peptone-hydrolyzing enzyme is of endocellular nature. Proof of this was obtained by comparing the peptone-splitting activity of the supernatant of an unautolyzed broth culture with the activity of a sterile solution prepared from the bacterial cells removed from the broth culture by centrifugation. The results of these experiments showed that while solutions of the intra-cellular substances of the bacteria actively hydrolyzed peptone, the bacteria-free supernatant fluid of unautolyzed cultures is practically devoid of peptone-splitting properties.

A slight activity exhibited by the bacteria-free filtrate of the broth culture can be referred to the liberation of the intracellular substances by the dissolution of the bacterial cells, a process which occurs rela-tively early in meningococcus cultures.

Comparison of Resistance to Heat and to Oxidation of the Maltase and Peptonase Enzymes of Meningococci.

In a previous paper on the oxidation of pneumococcus enzymes (4) it was pointed out that the activity of some of the more labile enzymes may be destroyed by oxidation processes which cause little loss in the activity of the more resistant enzymes present in the same mixture of intracellular substances. It was also shown that the relative order of resistance of the hydrolyzing enzymes to oxidation agrees with the order of heat resistance of the same enzymes. It seemed of interest, therefore, to determine if the same relation holds true for the maltase and peptonase enzymes of meningococci, both of which are present in the bacterial cell solution.

Facts illustrating the relative resistance of the meningococcus enzymes were obtained by the following experiment.

A portion of enzyme solution was heated for 10 minutes at 55°C., a period which had previously been proved sufficient to completely inactivate the maltase. Two

other portions of enzyme solution were exposed for 12 hours to different concentrations of H_2O_2. The initial concentration of H_2O_2 in the first tube was 150 mM; in the second tube, 25 mM. A part of the H_2O_2 was destroyed by the catalase and peroxidase of the meningococcus cell solution so that the concentration of the oxidizing agent during most of the oxidation treatment was considerably less than is represented by these figures.

For tests of peptone-splitting activity the following hydrolysis mixtures were prepared: (1) peptone plus untreated, or active enzyme; (2) peptone plus enzyme heated 10 minutes at 55°C.; (3) peptone plus enzyme exposed to 25 mM H_2O_2; (4) peptone plus enzyme exposed to 150 mM H_2O_2; (5) peptone plus enzyme completely inactivated by heat (boiled). A similar series was prepared to test the maltase activity, which was done by adding equal amounts of the above treated enzyme solutions to sterile maltose.

TABLE II.

Relative Resistance to Heat and to Oxidation Exhibited by the Peptonase and Maltase Enzymes of Meningococcus.

Treatment of enzyme	Peptonase activity (increase in NH$_2$-N, mg. in 100 cc.)	Maltase activity
	mg.	
Active (or untreated) enzyme solution...................	13.0	+
Enzyme solution heated 10 min. at 55°C................	9.8	0
Enzyme solution exposed to 25 mM H_2O_2................	10.2	0
Enzyme solution exposed to 150 mM H_2O_2...............	3.1	0
Enzyme solution heated 30 min. at 100°C. (control)......	0	0

The maltose-splitting was determined by the biological method described in the preceding paper.

The results of this experiment (Table II) show that the meningococcus maltase is completely inactivated by heating and oxidation treatment which is insufficient to inactivate all of the peptone-hydrolyzing enzyme. This difference in relative stability of the carbohydrate-splitting and peptone-splitting enzymes of meningococci is similar to that previously shown (3) to hold for the same enzymes of pneumococci.

DISCUSSION.

Evidence has been presented that the meningococcus cell contains an active enzyme which on separation from the bacteria suffices to

hydrolyze peptone to simpler peptides and amino nitrogen. The commercial peptone used as substrate in the demonstration of the enzyme represents a mixture of protein hydrolysis products which includes derivatives of varying degrees of complexity. Since living cultures of meningococci do not exhibit the marked proteolytic properties possessed by the so called "liquefying" bacteria, it is probable that the enzyme solutions used in the present investigation would not attack intact or native proteins to the same degree as peptone. In view of this evidence that the sterile bacterial solution acts more readily on the simple protein derivatives occurring in "peptone," it is advisable to adopt the term "peptonase" in referring to the active enzyme involved. This term has been employed by Avery and Cullen (5) to describe the enzyme or enzymes of Pneumococcus which hydrolyze the peptides of peptone to simpler products. The "peptonase" enzyme of Meningococcus is more resistant both to heat and to oxidation than is the maltase enzyme, just as the "peptonase" enzyme of Pneumococcus is more resistant than the carbohydrate-splitting enzymes of the same bacteria.

The peptonase enzyme is endocellular in nature and must be regarded as a heat-labile constituent of the meningococcus cell, which would be set free whenever lysis of the bacteria occurs either in artificial culture or in the tissues of an infected host.

SUMMARY.

The meningococcus cell contains a peptonase enzyme which hydrolyzes the peptides and similar constituents of commercial "peptone." The activity of this enzyme is independent of the presence of the formed bacterial cell. The peptonase enzyme is more resistant to heat and to oxidation than is the maltase enzyme of the same bacteria.

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STUDIES ON BACTERIAL ENZYMES.

III. Pneumococcus Maltase and Lactase.

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INTRODUCTION.

Intensive study of Pneumococcus at the Hospital of The Rockefeller Institute has resulted in a better understanding of the constituents of the bacterial cell. Among the active substances thus far studied may be mentioned, the endocellular hemotoxin (1), proteolytic enzymes (2), lipolytic enzymes (2), carbohydrate-splitting enzymes (2), bacteriolytic enzymes (2), and thermolabile cellular substances (3) concerned in oxidation and reduction processes; together with the carbohydrate soluble specific substance, and the antigenic protein fraction of the cell (4).

The carbohydrate-hydrolyzing enzymes previously recognized include the amylase, inulinase, and sucrase reported by Avery and Cullen (2) and the raffinase reported by Neill and Avery (5). The experiments reported here add maltase and lactase enzymes to the list of agents involved in the biochemical activities of living pneumococci.

While, as a general principle, it is true that hydrolysis to hexoses is a preliminary step in the acid fermentation of lactose and similar disaccharides by the more common bacteria, the assumption that this applies to all bacteria is based upon scant experimental evidence, and some authorities (5) (Kruse, Fischer, and others) have concluded that the rule is not universally applicable. A number of workers (6) have shown that the products of the fermentation of complex carbohydrates differ sometimes not only quantitatively, but qualitatively from those yielded in the fermentation of the component hexoses. Gayon and Dubourg (7) report differences in the products of the fermentation of

sucrose and of fructose which are especially difficult to explain if sucrose is hydrolyzed before being fermented by the types of bacteria they employed. Phenomena of this nature have been observed chiefly in "mixed acid" fermentations rather than in the type of acid fermentation induced by the "true" lactic acid bacteria most closely related to Pneumococcus. However, the existence of microorganisms inducing an unusual type of fermentation of the complex sugars makes it worth while to seek for experimental evidence of the hydrolyzing enzymes rather than to assume their presence from the fact that the disaccharide is fermented by the bacteria.

TABLE I.

Demonstration of Activity of Pneumococcus Maltase and Lactase.

Hydrolysis mixture		Hydrolysis mixture treated with		Change in pH due to fermentation of hexoses formed by pneumococcus enzymes
		Shiga dysentery bacilli	Typhoid bacilli	
		pH	pH	pH
Maltose	Active enzyme	7.0	No test	0.5
	Heat-inactivated enzyme	7.5	No test	
Lactose	Active enzyme	6.5	6.6	1.0
	Heat-inactivated enzyme	7.5	7.5	

EXPERIMENTAL.

Methods.

Enzyme Solutions.—Sterile extracts of pneumococcus cells were used to demonstrate the enzymes. The pneumococcus extracts were prepared by the method previously described (3). Broth cultures were centrifuged; the concentrated bacterial cells were collected and suspended in a small volume of the supernatant broth; the pneumococcus suspensions were frozen and thawed repeatedly to liberate the intracellular substances. The extracts were finally filtered through a Berkefeld candle. Hence, the pneumococcus extracts employed as enzyme solutions were not only sterile but were free from cell fragments.

Demonstration of Activity of Pneumococcus Maltase and Lactase.

The following experiment illustrates the fact that sterile extracts of pneumococci possess the property of hydrolyzing maltose and lactose.

0.2 cc. of a sterile solution of pneumococci was added to 3.0 cc. of sterile maltose and lactose solution. A control series was prepared with enzyme solution which had been inactivated by heat. Shiga dysentery bacilli, which attack neither maltose nor lactose, were used as fermenting agents in the detection of the hexose products of the enzyme action. Tests of the lactose hydrolysis mixtures were also made with typhoid bacilli.

The results given in Table I demonstrate that pneumococci possess an active maltase and an active lactase enzyme in addition to the previously described carbohydrate-splitting enzymes. It is interesting to note that the lactase enzyme is apparently more active than the maltase as indicated by the greater acid formation in the fermentation tests made upon the lactose hydrolysis mixture. Since the concentration of the pneumococcus solution was the same in both the maltose and lactose tests, this fact suggests that either the pneumococcus cell contains a more active lactase, or that the maltase enzyme if initially as active had deteriorated to a greater extent than the lactase at the time of the enzyme tests.

Comparison of the Activity of Different Carbohydrate-Splitting Enzymes of Pneumococcus.

The preceding experiment showed an apparent difference in the activity with which the sterile pneumococcus cell solution hydrolyzed maltose and lactose. It, therefore, seemed of interest to compare the apparent activity of other carbohydrate-splitting enzymes contained in the sterile cell solution with the activity of the maltase.

This comparison was made by the fermentation method used in the preceding experiment. Equal amounts of the pneumococcus solution were added to sterile solutions of maltose, lactose, sucrose, and raffinose. The hexose products of the enzyme action were detected by use of Shiga dysentery bacilli as fermenting agents. There is an objection to this method in such comparisons since the tests may involve differences in the ease of fermentation of the different hexoses (glucose, fructose, and galactose) yielded in the hydrolysis of the different sugars. However, these differences are almost always simply differences in rate of acid formation, which vanish when a greater time is allowed for hexose fermentation by the dysentery bacilli. The results of the experiment are summarized in Table II.

Without presuming to attach too precise a quantitative value to the data in Table II, one can yet be certain that the differences in acid

formation in the test solutions indicate differences in the degree to which the various sugars have been hydrolyzed by the pneumococcus enzymes.   The acid produced in the maltose mixture was unquestionably much less in quantity than that in any of the other test solutions. This indicates that the pneumococcus cell solution hydrolyzes maltose less actively than either of the other two disaccharides, and, more surprising, less actively than the trisaccharide, raffinose.   One cannot be sure whether these experimental results are due to a relative inability of pneumococcus cells of this particular strain to hydrolyze maltose or

TABLE II.

Comparison of the Activity of Different Carbohydrate-Splitting Enzymes of Pneumococcus.

Hydrolysis mixture		Change in pH due to acid fermentation of hexose formed by pneumococcus enzymes
Test sugar	Amount of pneumococcus cell solution	
		pH
Maltose	0.4 cc. enzyme solution	0.5
	0.1 cc. enzyme solution	0.3
Lactose	0 4 cc. enzyme solution	1.7
	0 1 cc. enzyme solution	0.6
Sucrose	0.4 cc. enzyme solution	1.4
	0.1 cc. enzyme solution	0.6
Raffinose	0.4 cc. enzyme solution	1.0
	0 1 cc. enzyme solution	0.5

whether the maltase enzyme is especially labile and is destroyed more readily during the manipulation involved in the preparation of the enzyme solution.   Maltose is apparently very readily hydrolyzed by the intact cells, for it seems to be as rapidly fermented as lactose in tests with the living bacteria.

Comparative tests of the heat lability and of the resistance to oxidation of the maltase, lactase, and sucrase were made on the enzyme solutions.   In all such tests, the cell solution lost its maltase activity entirely after a heating and oxidation treatment which was insufficient to destroy all of the active lactase and sucrase.   The

results, however, were not conclusive since they were complicated by the greater initial activity of the lactase and sucrase in the original cellular solution.

Experimental proof of most of the microbial carbohydrases has been made with yeasts and fungi, although sucrase has been described from a number of different bacteria. An extended search of the literature, however, reveals very few actual demonstrations of active maltase and lactase enzymes in sterile solutions prepared from bacteria. The results of the experiments described prove the existence of active lactase and maltase in sterile, filtered solutions of the intracellular substances of pneumococci. The recognition of these two enzymes adds to the list of cellular substances known to be involved in the metabolic functions of living pneumococci.

The pneumococcus is closely related to the saprophytic streptococci involved in the common souring of milk. Microbiologists (Jensen (9), and others) who are interested primarily in the biochemical activities of bacteria point out the fact that pneumococci and the lactic streptococci (*Streptococcus lacticus*) possess sufficient characters in common to justify their inclusion in the same biological group of "lactic acid streptococci." The lactic acid fermentation of lactose and other disaccharides is generally believed to involve two reactions: (1) the hydrolysis of the disaccharide to its component hexoses; (2) the conversion of the hexoses to lactic acid. With living cells it is impossible to demonstrate the products of the first reaction as only the final acid product can be detected. Like the intermediate products of many chemical reactions, the hexoses never accumulate in detectable amount, since the living bacteria convert them to acid as rapidly as they are formed from the disaccharide. Indeed, the actual proof that two reactions are involved rests upon the fact that of the two processes (hydrolysis and fermentation) effected by the living cell, only the hydrolytic property retains its activity in sterile filtered solutions of the intracellular substances. Apparently, the power of the bacterial cell to attack hexoses is most intimately bound up with its morphological integrity, since solutions of the cellular substances, although

exhibiting many of the activities of the intact bacteria, seem to be devoid of action upon glucose (9).

Pneumococcus, in its present state of adaptation, is generally considered to have no extended independent existence outside of the animal body and its association with carbohydrates would seem to be limited to the foodstuffs encountered during its growth in the mouth. It is interesting, therefore, to find that the strictly parasitic Pneumococcus contains an endocellular equipment of carbohydrases that rivals the generous endowment of purely saprophytic fungi.

SUMMARY.

Lactase and maltase are constituents of the pneumococcus cell.

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THE ETIOLOGY OF VERRUGA PERUANA.

By HIDEYO NOGUCHI, M.D.

The experimental investigation to be reported in this paper was made possible through the cooperation of Professor Oswaldo Hercelles, of the University of Lima, who was kind enough to obtain for me selected material for study, and I wish at the outset to acknowledge my indebtedness to him and my appreciation of his courtesy. I wish to express my thanks also to Professor E. Campodónico, of the University of Lima, through whose courtesy the material was forwarded from Lima to New York.

There are many similarities between the conditions known as verruga peruana and Oroya fever. Their geographical distribution, which is rather curiously limited to certain narrow valleys on the western slopes of the Andes Mountains, between 6° and 13° south latitude, is practically the same and is of the type shown by infections conveyed by certain ectoparasites. Fever and anemia are considerably more acute and severe in Oroya fever, but both are present in verruga. Verruga is predominantly a chronic infection, and its conspicuous feature is the characteristic nodular eruption on the skin which lasts for a period of a few weeks to one of many months. Oroya fever, on the other hand, while sometimes accompanied or followed by a similar skin eruption, is distinguished by an acute course of extreme anemia, during which a specific intracellular parasite, *Bartonella bacilliformis*, is abundantly present in the red blood corpuscles. This microorganism has rarely been seen in the blood in cases of benign verruga.

In 1910 Jadassohn and Seiffert[1] showed that verruga peruana could be transmitted to monkeys by local inoculation of suspensions

[1] Jadassohn and Seiffert, G., *Z. Hyg. u. Infectionskrankh.*, 1910, lxvi, 247.

of tissue from human verruga lesions, and their results have received confirmation from Mayer, Rocha-Lima, and Werner,[2] and from the Commission of the Harvard School of Tropical Medicine.[3]

Oroya fever has not thus far been directly transmitted to animals, but by means of a culture recently isolated from a fatal case of the disease[4] it has been shown that young *rhesus* monkeys (about 2,000 gm. body weight) are susceptible to infection with *Bartonella bacilliformis*, though usually not to the same degree as man, and that in the infected animals the parasite is demonstrable in its characteristic situation in the erythrocytes. In some animals which are less resistant to infection, the extreme anemia of Oroya fever has been reproduced.[5] The striking fact brought out in the inoculation experiments, however, was the dual nature of the infection induced, for characteristic signs of both Oroya fever and verruga were observed in the animals, with occasionally a marked favoring of one or the other type of disease, as the virulence of the strain became enhanced by adaptation to the experimental animal. Systemic manifestations—moderate or marked anemia, fever, and high titer of *Bartonella bacilliformis* in the blood, bone marrow, lymph nodes, and spleen—were in general more severe in animals inoculated intravenously or intraperitoneally, and death occurred only in animals so inoculated, but the invasion of the blood by the parasite was found to take place also after local inoculation. The tendency toward the production of verrucous lesions was more marked, as a rule, when the virus was introduced locally, and the lesions usually arose only at the sites of inoculation, as is the case when monkeys are inoculated directly with human verruga material.[1,2] In one instance, however, in which passage virus was injected both locally and intravenously, a spontaneous general eruption typical of severe human verruga arose on various parts of the skin, remote from the sites of inoculation. The skin lesions of the animals, whether local or

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[2] *Mayer, M., Rocha-Lima, H., and Werner, H., Münch. med. Woch., 1913, lx,* 739.

[3] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[4] Noguchi, H , and Battistini, T. S., *J. Exp. Med ,* 1926, xliii, 851.

[5] Noguchi, H., *J. Exp. Med.,* 1926, xliv, 697.

spontaneous in occurrence, in all instances yielded cultures of *Bartonella bacilliformis*, and stained sections of all such lesions revealed the parasites in large numbers in a characteristic endothelial situation.[5]

These findings, when taken in relation to the historical and epidemiological data with regard to the two conditions,[6] suggested very strongly that Oroya fever and verruga peruana were both due to *Bartonella bacilliformis*, the considerable variations in the manifestations of infection being a result of the differences in resistance among individuals or among different tissues of the same individual. It was desirable, however, to approach the problem of the etiology of verruga directly, by means of the cultural and experimental methods used for the isolation of *Bartonella bacilliformis* from Oroya fever.

For the purpose of comparative study subcutaneous nodules were removed from each of two cases of verruga in the Dos de Mayo Hospital, Lima, placed in tubes, and covered with citrate-saline solution (2 per cent sodium citrate in 0.9 per cent sodium chloride). The tubes were sealed, abundant air space being allowed, and were placed in the ship's refrigerator during the journey to New York, a period of 14 days. We were fortunate in having transportation so arranged that the tissues were shipped shortly after their excision from the patients and were received in the laboratory the same day that they arrived in New York (April 7, 1926). Each specimen was immediately examined microscopically, saline suspensions were made, and inoculated into culture media and experimental animals, and a portion of each tissue was fixed in Regaud's fluid for histological study.

The nodule of Case P. 5 measured about 8 × 12 × 6 mm. and was light pinkish in color, with the exception of a small portion to which the skin was still attached. The nodule was still firm, except for slight softening along the cut surfaces. The citrate solution was slightly turbid, owing to liberation of tissue elements, but there was no putrefactive odor.

Minute Gram-negative bacilli were present, occurring singly, in pairs, and in larger groups, as well as in masses (Fig. 18). Sections stained with Giemsa's and Gram's solutions showed masses of the organisms scattered through the tissue (Fig. 18), the general structure of which was still well preserved (Fig. 17) notwithstanding loss of cellular elements through autolysis.

[6] Odriozola, E., La maladie de Carrion, Paris, 1896.

The nodule from Case J. 45 was not as well preserved. The tissue was soft and friable, but there was no sign of bacterial putrefaction. It contained numerous Gram-negative bacilli similar to those found in the specimen from Case P. 5 and in addition a few small Gram-positive diplococci. In sections the bacilli were found in the nodular tissue and the cocci near and on the skin.

Two species of minute Gram-negative microorganisms were isolated from the tissue of Case P. 5, one indirectly, from monkeys inoculated with suspensions of the tissue, and one by direct cultivation. The former is pathogenic for young *Macacus rhesus* monkeys and in every respect has proved identical with the strain of *Bartonella bacilliformis* previously obtained from the blood of a case of Oroya fever. The latter, although present also in the J. 45 tissue, as shown by cultivation experiments, is apparently a non-specific invader; it has failed to induce characteristic local or systemic infection, and it grows within 24 hours on ordinary agar slants, while the pathogenic organism grows slowly and only on leptospira medium or blood agar slants. Our interest, therefore, is in the pathogenic strain obtained by inoculation of the P. 5 material.

Transmission of Verruga Peruana to Monkeys with the Excised Nodular Tissue of Case P. 5.

Transmission of verruga peruana to monkeys, as stated earlier in this paper, has repeatedly been accomplished by previous investigators. In the present instance, however, the tissue had been excised from the patients more than 2 weeks previous to inoculation.

One young and one adult *Macacus rhesus* were available on April 7. The young one was used for inoculation of the more promising material, that is, the P. 5 nodule. Another young monkey, obtained on April 10, was also inoculated with the P. 5 material. Owing to the scarcity of suitable animals, the inoculations with the J. 45 material could not be repeated.

The suspension of the P. 5 nodule induced definite systemic or local infection in both animals inoculated; the full grown monkey receiving inoculation of the J. 45 material showed no symptoms of infection.

M. rhesus 33, inoculated Apr. 7, 1926, with a saline suspension of the nodule P. 5, intradermally on the shaved right eyebrow and by scarification on the left eyebrow.

2 cc. of the suspension were also injected into the saphenous vein of the right leg. After 48 hours there was a rise of temperature (Chart 1) which lasted for 2 days, but blood taken at this time yielded no growth. On Apr. 14 the temperature rose again and remained high for 6 days. Blood taken during the fever (Apr 16) and inoculated into leptospira medium yielded, in a 1:10 dilution, pure growth of a microorganism indistinguishable from *Bartonella bacilliformis*. The sites of intradermal inoculations on the eyebrow showed some induration at this time, but they never increased in size and receded within a few weeks. The lymph glands in the inguinal and axillary regions became markedly enlarged about Apr. 16. Blood cultures made on two successive occasions, May 27 and June 20, 1926, both gave pure cultures of the *Bartonella*-like organisms.
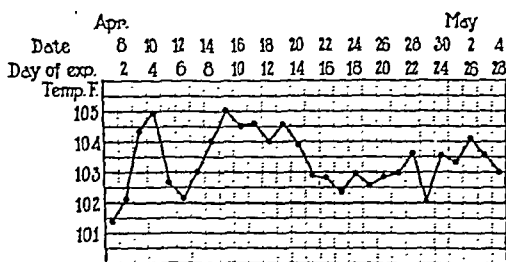
CHART 1. *M. rhesus* 33.
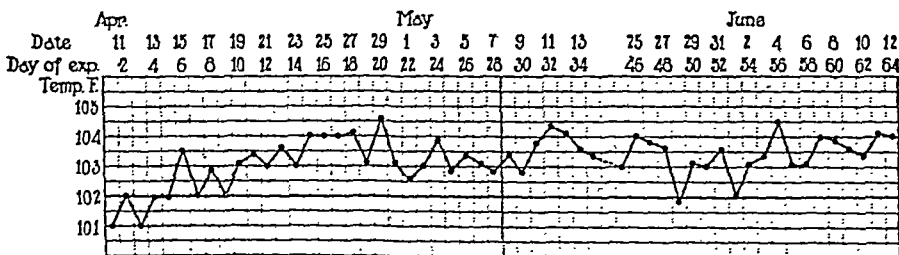
CHART 2. *M. rhesus* 34.

M. rhesus 34, inoculated Apr. 10, 1926, with the same material and in the same way as *M. rhesus* 33. A moderate rise of temperature occurred 14 days from the time of inoculation (Chart 2), and the animal remained febrile for 4 days. Blood cultures made on Apr. 28, 1926, yielded a pure growth of the same organism as had been isolated from *M. rhesus* 33. At this time the lymph glands had become markedly enlarged, but no local reactions were noticeable. Blood taken on May 12 and on May 27 was culturally positive in a 1:10 dilution. At the beginning of June a large subcutaneous nodule at the middle portion of the tail was noticed

(Fig. 10), and on June 18 it was excised * It showed the typical histology of ver-
ruga tissue (Fig. 11) and yielded a pure culture of the same organism as had been
obtained from the blood. Cultivation of the blood was again successful on June
21, this time with a dilution of 1:100.

The foregoing experiments demonstrate the infectivity of the
verruga nodule which had been kept at refrigerator temperature for at
least 2 weeks, and the association of infective power (as evidenced
by febrile reaction and the production of a metastatic local verruga
lesion) with a definite microorganism recoverable by culture both from
the blood of the animals and from the local lesion. Blood counts
revealed no anemia in either animal, and in neither instance did the
blood films contain a sufficient number of organisms to be detected
microscopically.
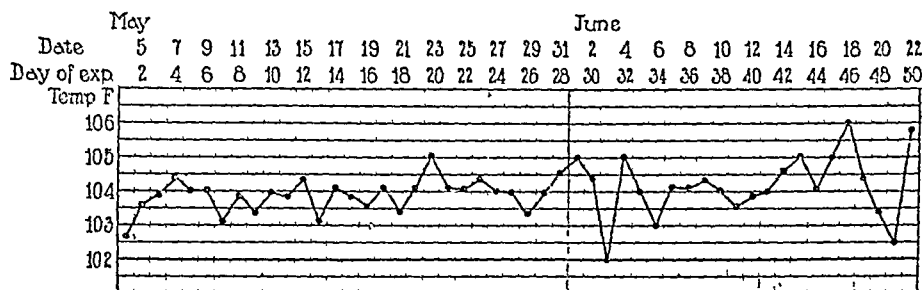
CHART 3. *M rhesus* 41

The pathogenicity of the culture obtained from the blood of *M.
rhesus* 33 on April 16 was tested by inoculation of *M. rhesus* 41. As
the protocol shows, the culture gave rise not only to a local reaction
but to a systemic infection of pronounced severity.

M. rhesus 41 (Fig. 1) inoculated May 4, 1926, with culture derived from the
blood of *M. rhesus* 33 and grown for 18 days on leptospira medium. The material
was inoculated on the right eyebrow and abdomen intradermally and on the left
eyebrow and abdomen by scarification. An irregular remittent febrile reaction
(Chart 3) began to be manifest 72 hours from the time of inoculation and continued
during the 2 months of observation. Induration became noticeable at the site
of intradermal inoculation on the right eyebrow after 14 days. The lesion had the
gross appearance of a typical verruga nodule, and histological examination of the

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* All operations were carried out under ether anesthesia.

excised tissue showed the characteristic granulomatous structure (Figs. 4, 12) and the presence of bacilliform organisms in the endothelial cells (Fig. 13). A suspension of the nodular tissue yielded pure cultures of the organisms in a 1:10 dilution, as did also blood withdrawn on May 21. On May 27 the blood titer by culture was 1:10,000, and on June 3, 1:100,000. The nodules on the eyebrow rapidly increased in size, becoming scarlet-red and protruding (Fig. 1). Blood films made on June 3 showed numerous bacilliform bodies in the red corpuscles, the organisms being present in the largest numbers so far observed in *rhesus* monkeys (Figs. 2, 14). Small nodules developed at the sites of intradermal inoculation on the abdomen 40 days after inoculation; at that time the nodules on the eyebrow measured about 10 × 12 mm.

Blood counts made by Dr. J. H. Bauer showed a gradual diminution of red corpuscles and hemoglobin and an unusually high leucocytosis:

	Erythrocytes per c.mm.	Hemoglobin	Leucocytes per c.mm.
		per cent	
May 4 (day of inoculation)................	5,088,000	80	
" 14 (10 days after inoculation)...........	4,632,000	70	
June 3 (30 " " ")...........	4,760,000	55	17,600
" 15 (42 " " ")...........	4,800,000	40	23,000
" 23 (50 " " ")...........	3,392,000	30	43,000
" 25 (52 " " ")...........	3,460,000	25	40,000
" 28 (55 " " ")...........	3,552,000	25	37,800

The reduction in hemoglobin, it will be noted, was relatively greater than that in the number of red cells, a phenomenon so far not observed by us in animals infected with the strain of *Bartonella bacilliformis* from Oroya fever.

Blood smears stained on June 25 revealed a peculiar appearance of the intracorpuscular parasites, which were still present in rather large numbers, but stained reddish rather than violet, appeared thinner, and in some instances were fragmented. The general features of the organisms suggested that they were undergoing degeneration (Figs. 3, 15). Blood taken at this time yielded cultures in dilution of 1:10, but not in 1:100. A week later no intracorpuscular parasites could be demonstrated, the animal was more active, and the nodules had decreased somewhat in size. Conditions seemed to indicate the development of a state of immunity. No cultures could be obtained from the blood after June 25. On Aug. 11 there was still anemia (red blood cells 3,362,000, hemoglobin 50 per cent), but on Sept. 14 the number of red cells had increased to 4,336,000.

The nodule excised on May 19 and the blood withdrawn 2 days later were inoculated into two *rhesus* monkeys. As the protocols show, both materials gave rise to local lesions and also to systemic infection.

M. rhesus 42, inoculated May 19, 1926, with a saline suspension of the nodule from the eyebrow of *M. rhesus* 41, on the left eyebrow and left abdominal wall by intradermal injection and on the right eyebrow and right abdominal wall by scarification. This animal had fever for only a few days (Chart 4) during the 45 days of observation, but cultures made with the blood on June 2 and 14, 1926, gave pure growth of the bacilliform organisms in dilutions of 1:10 and 1:1,000 respectively.

Small reddish indurated areas along the lines of scarification on both eyebrow and abdomen became noticeable on June 1 (Fig. 6), and 10 days later the scarified areas appeared as linear rows of eruptions (Figs. 7, 8). Within another 10 days the adjacent lesions had become confluent (Fig. 9) but the transverse lines were still well separated. The intradermal inoculations in this instance, contrary to the usual case, failed to induce any lesions
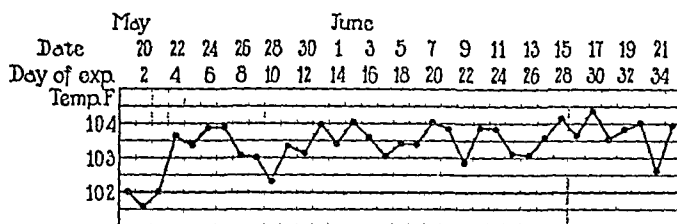
CHART 4.   *M. rhesus* 42.
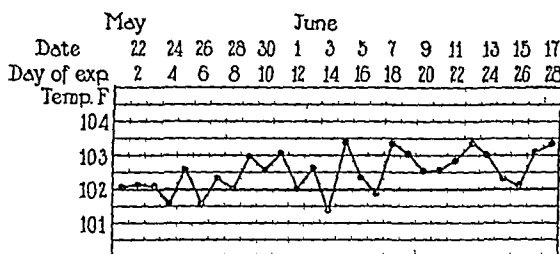
CHART 5.   *M. rhesus* 43.

The lesions had all healed by Aug. 6, 1926.

M. rhesus 43, inoculated May 21, 1926, under ether anesthesia, with the citrate blood withdrawn on the same day from *M. rhesus* 33. Intradermal inoculations were made on both eyebrows and on both sides of the abdomen. Blood was also applied to scarified areas on the eyebrows and abdomen, and 0.5 cc. was injected intravenously.

The animal showed no febrile reaction at any time during the 45 days of observation (Chart 5). Blood cultures made on June 26 were negative. Small nodules, 2×2 mm., appeared at each of the two sites of intradermal inoculation within about 24 days. They showed no change during the ensuing 10 days but

subsequently gained in size rather rapidly. They were situated subcutaneously, were perfectly round, and had a semitransparent, bluish, pearl-like color. On July 1 there were noticed about the periphery the minute red streaks which *Bartonella* lesions of this type show just before vascularization. One of the nodules excised at this time (July 1) yielded cultures in a dilution of 1:100,000. By Aug. 6, 1926, the lesions had all healed.

The microorganism obtained in culture from a monkey inoculated with human verruga tissue (Case P. 5) was capable, therefore, of inducing in *rhesus* monkeys a local and systemic infection the manifestations of which were in all respects similar to those obtained by inoculation of the strain of *Bartonella bacilliformis* from Oroya fever, *i.e.*, a prolonged course of irregular, remittent fever, during which the intracorpuscular parasites are present in the blood, and a skin lesion characterized by extensive proliferation of endothelial cells and by the presence of the parasites in the cytoplasm of these cells. The infection in the second passage animal (Monkey 41) was of the severe type; there was absolute anemia and hyperleucocytosis, and the local lesions were very large. The organisms were present in large numbers at one period of the disease, but only degenerated forms were found at a later stage. The blood from which the culture was obtained, however, induced only a mild, slowly developing infection, with no fever.

The local inoculation of nodular tissue from Monkey 41 into Monkey 42 (third passage) induced a severe local reaction and a febrile reaction of brief duration, during which the blood culture titer was as high as 1:1,000. As stated previously, direct microscopic detection of the parasites in the blood was unsuccessful in the case of mild infections. The cultural procedure was by far the most delicate means of diagnosis.

Cultural and Morphological Characters of the Microorganisms from Verruga.

The character of the lesions of *M. rhesus* 41 and the microscopic findings in the blood of this animal appear to leave no doubt that the organism injected was identical in pathogenic properties with the strain of *Bartonella bacilliformis* previously obtained from Oroya fever, a conclusion which is further substantiated by cultural and morphological study.

The first culture was obtained on the so called leptospira medium, which was inoculated with various dilutions of citrated blood of *M. rhesus* 33 and kept at 25°C. Growth became recognizable macroscopically within a week, the uppermost layer of the medium showing some grayish, minute particles; these gradually increased in number during subsequent weeks (Fig. 19). Under the dark-field microscope these particles were seen to consist of numerous tightly packed masses of irregular minute bacillary bodies. Individual organisms were occasionally seen, but the chief characteristic of the organisms is their constant tendency to form firm aggregates difficult to disperse (Fig. 21). Free flagella are seen in the agar mass.

On the surface of blood agar round raised colonies of variable size, the smallest almost microscopic (Fig. 20), the largest having the appearance of fine granules, are formed within about 6 to 8 days and after a few days reach 1 mm. or more in diameter, but seldom 2 mm. A light grayish color is noticeable when the light falls obliquely on the culture. The colonies are firm and are peeled off the agar readily by a platinum loop. It is difficult to break them up in fluid to form a uniform suspension. Under the dark-field microscope masses of agglomerated organisms are seen to predominate (Fig. 22); a few single or paired individuals may be found, and these are usually motile. Locomotion is in one direction only, but rotatory movement is also seen.

The organisms are Gram-negative, pleomorphic, and stain unevenly with Giemsa's solution. When stained a long time they appear reddish and ill defined in contour; they stain much less intensely than most bacteria. Special staining reveals two or more flagella attached to one end of the organism (Fig. 24), the length of the normal flagella varying from 2 to 5 μ. Abnormal detached flagella may reach a length of 20 to 30 μ.

Individuals in young cultures are more uniform in size and have a definite contour. Short rods, 0.3 to 0.4 μ wide and 0.45 to 1.5 μ in length, predominate. In older cultures the individuals are much more irregular in size and form, and numerous granular elements measuring less than 0.2 to 0.5 μ are found in masses; these stain intensely and have very indefinite outlines, the appearance simulating that of degenerative changes   Exceptionally long rods 2.5 to 3 μ may be found. There is no polar staining.

The optimum temperature for growth is 25–28°C.; at 37°C. growth ceases within 4 to 5 days. The optimum reaction of the medium lies between pH 7.8 and pH 8.

For the cultivation of this organism the addition of animal blood or serum and hemoglobin to solid or semisolid media is essential. No satisfactory fluid medium has so far been found. The organism is an obligate aerobe. The cotton plugs of culture tubes may be impregnated with paraffin, but sealing them hermetically with sealing wax prevents growth.

Like the strain of *Bartonella bacilliformis* from Oroya fever, the organism is non-spore-forming and hemophilic. It is provided with

TABLE I.

Anti-Oroya serum	Antigens	
	Oroya	Verruga
	0.5 cc.	0.5 cc.
cc.		
0.1	++++	++++
0.01	++++	++++
0.001	++	—
0.0001	—	—
0	—	—

++++ = complete fixation.
++ = 50 per cent hemolysis.
— = complete hemolysis.

several unipolar flagella of the characteristic bacterial type. A distinct cell wall, such as is seen in bacteria, can be recognized in young, actively motile forms.

The organism does not ferment any carbohydrate so far tested. Red corpuscles contained in the culture medium are not hemolyzed in the course of its growth. No putrefactive process seems to occur in the culture. Slight growth may be obtained on Loeffler's serum medium, but no liquefaction is observed.

Serological Identification of the Verruga Strain.

The morphological, cultural, and pathogenic properties of the verruga strain resemble so much those of the strain of *Bartonella*

bacilliformis obtained from a case of Oroya fever that the identity of the two organisms appeared extremely probable. Preliminary serological tests confirmed this conclusion.

A rabbit was repeatedly inoculated intravenously with saline suspensions of cultures of *Bartonella bacilliformis* during a period of 4 months, 1 to 2 cc. being injected at intervals of 4 to 5 days, and the serum obtained from the animal was tested for complement fixation with its homologous strain and also with the strain from verruga. Saline suspensions of colonies removed from the surface of blood slant cultures after 14 days growth at 25°C., and washed twice with saline solution, served as antigens. The suspensions were rather granular in appearance, owing to the difficulty of breaking up the colonies. To 0.5 cc. of the antigen (this quantity did not bind complement) were added the immune serum in quantities of 0.1, 0.01, 0.001, or 0.0001 cc., and 0.04 cc. guinea pig serum as complement. Two hemolytic units of anti-sheep amboceptor were used. Controls with normal rabbit serum were made in each instance. The total volume in each case was made up to 1.2 cc. with saline solution. The results are shown in Table I.

The results of the complement fixation tests show that there is an undeniably close serological relationship between the two strains, although the immune serum reacted more strongly with the Oroya than with the verruga strain.

SUMMARY AND CONCLUSIONS.

A saline suspension of a subcutaneous nodule excised from a verruga patient, and kept in the refrigerator for 14 days, on inoculation into two young *Macacus rhesus* monkeys (Nos. 33 and 34) induced irregular febrile reactions and enlargement of the lymph glands, and in one instance a subcutaneous nodule arose, independently of direct inoculation, on the tail. A microorganism has been isolated from the blood of both animals, and from the experimental nodule, which in pathogenic properties and in cultural and morphological characteristics is indistinguishable from the strain of *Bartonella bacilliformis* isolated from a case of Oroya fever.

The spontaneous skin lesion of Monkey 34 and the subcutaneous nodules induced by intradermal inoculation of cultures of the microorganism were histologically typical of experimental verruga lesions in monkeys and identical with the skin lesions induced in monkeys by *Bartonella bacilliformis*. The organism, like *Bartonella bacilliformis*, is an intracellular parasite, being found in the cytoplasm of the prolif-

erating endothelial cells of the lesions and in the erythrocytes of the blood.

The same variations in the manifestations of disease which have been noted in experimental infection with *Bartonella bacilliformis* were observed in the experimental verruga infection. In the second passage (Monkey 41) the infection induced by local inoculation of cultures was severe both locally and constitutionally and was accompanied by marked anemia. The organisms were found in the red cells in large numbers. In the third passage the systemic infection was less severe, but the local lesions were more striking.

Detection of the parasites in the blood is far more certain by the cultural method than by microscopic examination, the latter procedure being successful only in rather severe infections. The result of blood culture is therefore the decisive method in the final diagnosis of the disease.

Preliminary serological study shows that the organism isolated in the present instance from the skin lesion of a verruga patient and that previously obtained from the blood of a case of Oroya fever belong to the same serological group.

The data obtained justify the conclusion that verruga peruana is caused by *Bartonella bacilliformis*. They also definitely establish the fact that the inoculation of blood or sanguineous exudate from lesions of verruga peruana is capable of inducing in susceptible individuals a severe febrile systemic infection, such as that to which Carrion succumbed. The designation "Carrion's disease"[6] is therefore the appropriate one for both forms of the infection.

Bartonella bacilliformis may be regarded as a bacterium, since it has the essential features of that group of microorganisms.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. *M. rhesus* 41, showing three verruga nodules on the right eyebrow 30 days after intradermal inoculation. The two nodules on the inner side had arisen at the site of removal of the initial nodule 16 days previously. Natural size.

FIG. 2. Verruga organisms in the red corpuscles of *M. rhesus* 41, 30 days after inoculation. × 1,500.

FIG. 3. Verruga organisms in the red corpuscles of the same monkey 52 days

after inoculation. They took a more reddish stain at this time and were less definite in outline, probably because they were in process of degeneration. Anemia was also most marked at this time. Subsequent attempts to find the parasite in the blood were unsuccessful. × 1,500.

FIG. 4. Verruga organisms in the nodule of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after fixation in Regaud's fluid. × 1,500.

FIG. 5. Verruga organisms in the nodule of a patient suffering with verruga peruana. Courtesy of Professor R. P. Strong. Giemsa's stain, after fixation in Zenker's fluid. × 1,500.

FIG. 6. Early lesions of experimental verruga in *M. rhesus* 42, 16 days after inoculation of a scarified area on the right eyebrow with a suspension of the nodule from *M. rhesus* 41. Natural size.

FIG. 7. Same lesions 23 days after inoculation.

FIG. 8. Early lesions on the scarified skin of the abdomen of the same monkey 16 days after inoculation. Natural size.

FIG. 9. Same lesions 23 days after inoculation.

FIG. 10. Subcutaneous nodule which arose spontaneously on the tail of *M. rhesus* 34, 59 days after the animal had been inoculated with a suspension of a nodule of the verruga patient P. 5. Natural size. For histological appearance of the nodule see Fig. 11.

PLATE 3.

FIG. 11. Histological appearance of the subcutaneous nodule on the tail of *M. rhesus* 34, 69 days after inoculation. (The gross appearance of the nodule is shown in Fig. 10.) Giemsa's stain after fixation in Regaud's fluid. × 152.

FIG. 12. Histological appearance of the nodule on the eyebrow of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after Regaud's fixation. × 152.

FIG. 13. Verruga organisms in the nodule of *M. rhesus* 41. Same section as shown in Fig. 12. × 1,000.

FIG. 14. Verruga organisms in the red blood corpuscles of *M. rhesus* 41. Eight different fields have been placed side by side. In some of them are seen red corpuscles containing one or more organisms. Blood films made 30 days after inoculation. Giemsa's stain. × 1,000.

FIG. 15. Blood of the same monkey 52 days after inoculation. The organisms appear somewhat degenerated but quite numerous. Giemsa's stain. × 1,000.

FIG. 16. *Bartonella bacilliformis* in the blood of an Oroya fever patient, for comparison (Patient S. A. 15, case from which *B. bacilliformis* was cultivated). Giemsa's stain. × 1,000.

PLATE 4.

FIG. 17. Verruga nodule from Case P. 5, fixed in Regaud's fluid and stained with Giemsa's solution, 14 days after removal from patient. The characteristic verrucous structure is still recognizable, although many cells have undergone degenera-

tion. The dark masses of irregular size are aggregates of minute bacilliform organisms. × 152.

FIG. 18. The same section as that shown in Fig. 17 but under a higher magnification. × 1,000.

FIG. 19. Appearance of colonies of the verruga organisms in the upper portion of a tube of leptospira medium. 28 days old at 25°C. Natural size.

FIG. 20. Appearance of colonies of the verruga organisms on the surface of horse blood agar slant, 12 days old at 25°. Natural size.

FIG. 21. Dark-field view of the culture shown in Fig. 19. × 1,000.

FIG. 22. Dark-field view of the culture shown in Fig. 20. × 1,000.

FIG. 23. The verruga organisms from a blood agar slant culture, 11 days old at 25°C. Giemsa's stain. × 1,000.

FIG. 24. Flagella of the verruga organisms. Zettnow's stain. × 2,000.

Intradermal inoculation, 30 days.

Scarification, 16 days.

Blood, 30 days.   Blood, 52 days.

Scarification, 23 days.

Eyebrow nodule, 14 days.

16 days.   23 days.

Human nodule.   Nodule on tail.

PLATE 3.

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OF

EXPERIMENTAL MEDICINE

A METHOD FOR REPEATEDLY SAMPLING THE BLOOD OF THE PORTAL VEIN IN HEALTHY ANIMALS.

BY M. A. BLANKENHORN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Medical Research Laboratory of Lakeside Hospital, Cleveland.*)

A method to obtain samples of the blood of the portal vein at will would be of extreme value for the study of the phases of intestinal absorption and of liver problems not to mention others. The method here described was devised during the course of a study of the reabsorption of pigments of biliary origin for which the usual laboratory methods entailing laparotomy had proved inadequate. While this method does not permit sampling entirely at will, it does permit repeated tapping of the vein in the healthy and unanesthetized animal.

The plan pursued is to place a silver tube within the body of the animal. For weeks or months after the animal has recovered from the operation this tube will serve as a guide for a puncture of the portal vein.

To be successful as a guide to tapping the portal vein the tube must be in contact with it at one end. The other end must be palpable beneath the skin and easily entered by a needle large enough for the withdrawal of blood. The device must be well tolerated by the animal and do no injury to the abdominal viscera.

Such a cannula was made of silver in the form illustrated in Text-fig. 1. For convenience in installing it was made in three parts.

A is a saddle-shaped plate which spans the portal vein and is held there by large silk threads. *C* is a plate with a nipple on it which is fastened into the abdominal wall near the costal margin so that it is covered only by skin. *B* is a hollow stem that connects *A* and *C* by screwing into both these parts when they are in place. This stem is fitted with a right hand screw on one end and a left hand screw at the other so that turning in the one direction engages both the end

pieces (*A* and *C*) and draws them closer together. This screw arrangement not only facilitates assembling the apparatus but regulates the length of the cannula so that it will fit dogs of various sizes.

In practice the details of operation to install the cannula are as follows: Under ether, a midline incision is made from the xyphosternum to one-third the distance to the pubis; self-retaining retractors are put in and wide retraction made. A sand bag under the lumbar spine increases the exposure and large moist sponges are packed into the abdomen to keep the intestines out of the field. The portal vein in the dog is retroperitoneal in about one-half its circumference so a small amount of dissection is required to permit the saddle *A* to span the vein without tension. When the dissection is done two very thick silk threads are passed entirely under the vein at the cephalic and caudal ends of the dissection. These threads are quite long and both ends of each are brought up and out of the inci-
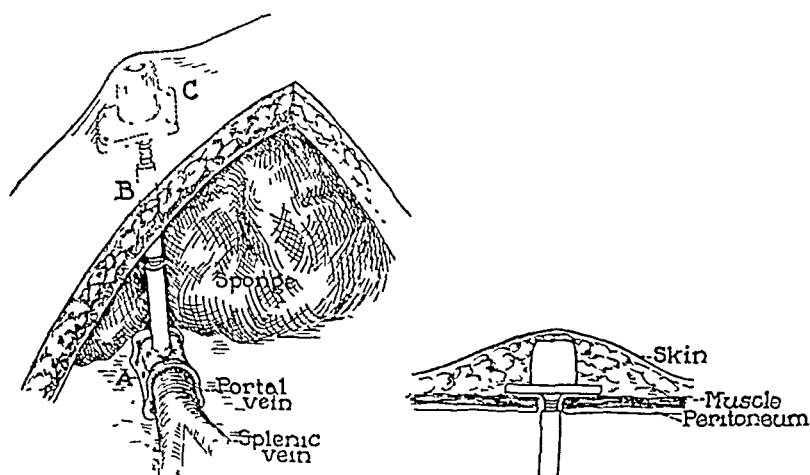
TEXT-FIG. 1.

sion. Each thread is now put through a hole in the four corners of the plate and the plate, held by a hemostat, is lowered along the threads and into place. When it is in place the opposing threads are tied over the top of the saddle. These ligatures keep the saddle in place without compressing the vein at any point.

The plate carrying the nipple *C* must next go in. To guard against the infection that commonly develops around foreign bodies the skin must not be injured and the button must be out of the line of the main incision. To make a bed for the button, the right belly wall is everted as far as possible, with the retractor out. The end of the ninth costal cartilage is clipped off and an incision made from within outwards through the muscles in the line of the ribs, care being taken not to penetrate the skin. With the finger in this incision a pocket is made by blunt dissection of the subcutaneous fascia that will be large enough to contain the plate. When the bed is prepared the piece of silver is pushed through the muscle

incision and held by an assistant, while the operator closes the button-hole-like incision of the muscles with heavy mattress sutures of silk. It is imperative to have this repair so well done that there is no strain on any thread and that the plate retains its subcutaneous position.

It was found necessary to employ a rather large plate to guarantee a firm and permanent fixation to the parietal wall. The nipple upon the plate must also be of such size that the skin is lifted slightly by it, since otherwise the local swelling incident to the healing process may make it difficult to find. Two of the experiments recorded as failures were unsuccessful because the nipple could not be found with a needle even though the plate was firmly fixed.

With the two ends of the apparatus now in place an assistant grasps each with a hemostat to keep them in proper alignment while the operator screws in the connecting stem with his fingers, to what appears to be the proper length for an avoidance of tension when the animal is once more upon its feet. The abdomen is then closed in three layers with silk. The entire operation must be done with strict surgical care against infection for the retained foreign body predisposes to localized peritonitis or abscess.

When the animal is well recovered and the incision healed, which is usually within a week, the tappings can be begun. If the length of the stem of the silver tube is correct for the animal, and there is no strain on the vein or on the abdominal wall, the device is well tolerated, and the animal appears normal. If free from infection the skin fits flatly over the button. Such serum as may exude about the plate has free access to the abdomen and drains away.

The technique of tapping is as follows: The animal is placed on the left side, and the skin over the button is shaved and swabbed with iodine. A No. 17 lumbar puncture needle with stylette is thrust through the skin and down the cannula until close to the vein, when the stylette is withdrawn and a syringe attached on the needle. With suction made the needle is now advanced till it enters the vein, when blood flows into the syringe. The distance from skin to vein must already have been marked on the needle. It will be known from the length of the cannula as installed. A convenient aspirating apparatus is a 20 cc. Record syringe fitted with a rubber stopper and tube so that the operator can make suction with his mouth while manipulating the needle. It is well to have a little oxalate solution in the syringe and the needle, for clotting occurs very readily, partly on account of the length of the needle and partly because blood or serum may enter the needle from the cannula before the vein is entered. Precautions to guard against infecting the vein at the time of the tapping will prolong the usefulness of the device.

To employ a needle with stylette complicates the process of puncturing the vein and doubtless consumes time at a critical moment when clotting is most likely to occur, but on the other hand the stylette keeps the needle relatively free from the clotted lymph or fibrin that fills the lumen of the cannula. A further detail in construction of the cannula not shown in the illustration is the presence of minute holes in the walls of the cannula stem. These holes permit the escape of fluid or fibrin when a needle is thrust into the cannula.

Thus far the apparatus has been installed in thirteen dogs. Of seven instances in a series at Lakeside Hospital, Cleveland, three were successful, and from one to seven specimens of blood were obtained from each dog. One animal was tapped $7\frac{1}{2}$ months after operation. Of six instances studied at The Rockefeller Institute four were successful and two unsuccessful. The form of the apparatus pictured was developed during the course of work at the Institute.

The chief limitations of the method are referable to thrombosis of the vein. This is a result of the contact of metal with the vein, the continuous movement of the cannula upon it with the breathing, and the repeated trauma from the aspirations. The thrombus which gradually develops does not entirely obstruct the vein but tends to block the end of the needle while permitting blood to flow round about it.

It is probable that celluloid will prove better than silver as a material from which to make the cannula. This material is apparently better tolerated than silver and had a certain amount of flexibility, yielding to respiratory movements with less trauma to the vein. The most successful series of tappings were accomplished with a celluloid cannula, at the Lakeside Hospital. This cannula eventually came apart by some solvent working on its cemented joint.

It is highly probable that methods to sample blood from deep lying veins have been described but the writer is aware of but one such in the literature. London[1] described a method that involved intubation of the splenic vein with a glass tube which was continued through the abdominal wall with rubber and kept closed with a dressing. The success of his arrangement was not reported upon.

The writer is indebted to Dr. P. D. McMaster of The Rockefeller Institute and to Dr. Graham and Dr. Beck of the Department of Surgery of Lakeside Hospital for assistance in these experiments.

SUMMARY.

A method is described for repeatedly sampling the blood of the portal vein in healthy animals. The method has been employed in thirteen dogs with success in eight.

[1] London, E., *Quart. J. Exp. Physiol.*, 1923, suppl.. 173.

ON THE ABSORPTION OF BILE PIGMENTS FROM THE INTESTINE.

By M. A. BLANKENHORN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Medical Research Laboratory of Lakeside Hospital, Cleveland.)

Clinical and experimental studies of the distribution and elimination of bile pigments in man have furnished valuable information as to the state of the liver and the function of bile ducts. Like studies aimed at an understanding of the condition of the blood with special reference to hemoglobin economy have not been so successful although it is well understood that there is an essential relationship between bile pigment and blood pigment (1). That blood destruction finds expression in bile pigment formation (2) and that true bile pigment, as distinct from that of extraneous origin, has no other source than hemoglobin (3) are facts now generally accepted; but as yet no certain quantitative relationship between the two has been established. The chemical process, taking place within the body, which leads to a conversion of one pigment into the other is entirely unknown and other factors less fundamental in nature interfere with our using bile pigment, or any of its derivatives, as a means to measure blood destruction. Among the most important of these factors is the possible reabsorption of bile pigments from the intestine, a reabsorption which might change the bile output or add to the store of hemoglobin. There is much clinical and some direct experimental (4) evidence that such reabsorption does occur. But the final proof, namely a demonstration of the presence of bile pigments in the blood or lymph coming away from the intestine and its removal by the liver, has been wanting. To supply this evidence has been the object of the present investigation.

The blood of the portal vein has been examined frequently for bile pigments, but always under the complex conditions which result from opening the abdomen; and no bile pigments have been found by any observer.

The method I have employed here is new and makes the portal vein accessible to puncture almost at will, weeks after the healing of the operative wound[1] necessary for the introduction of the puncture apparatus. To render the portal vein thus accessible a hollow silver tube is placed within the abdomen, one end being fixed to the vein while the other end lies beneath the skin of the belly wall where it is easily found on palpation prior to introducing a needle. The needle is thrust through the skin, through the tube directly into the vein. The complete details of this method are given in another paper.

In the present study seven dogs were successfully operated upon and furnished nineteen different specimens of portal vein blood to be tested for bile pigments. Six of the animals were also operated for "altercursive intubation" of the common bile duct after the manner of Rous and McMaster (5), so that the pigment content of the intestine could be controlled at will. Both operations were done in each instance at a single sitting.

When the operated animals were well recovered and appeared to be healthy, they were bled from jugular vein and portal vein at the same occasion and the specimens examined for bile pigments. The animals equipped with "altercursive intubation" were, after preliminary bleeding, adjusted for bile collection so that no bile entered the intestine. Stools and urine were collected daily and examined for bile pigment. When stools were entirely acholic, the animal was again bled from portal vein and jugular vein. Following this bleeding, bile was permitted to return to intestine and when stools showed the return of pigment bleeding was done again. In accord with this plan portal vein blood and jugular vein blood were compared as to their bile pigment content under conditions of choice in so far as there was or was not bile within the intestine. In but one dog were we able to get portal blood of all three phases in the same animal.

Blood specimens were tested for bilirubin by the diazo reaction, but quantitative tests were not possible on account of the minute quantities found. Urobilin was tested by the Schlessinger fluorescence method and quantitative determinations made according to a plan

[1] Ether anesthesia was used in every instance where an animal was operated upon, and anesthesia continued throughout the operation.

proposed by Elman and McMaster (6) and changed somewhat to deal with very small amounts as found in blood and lymph.

In brief these animals at no time showed any bilirubin in portal vein blood or jugular blood, nor even any yellow coloring of the plasma. On the other hand urobilin was always present in portal vein blood whenever there was bile in the intestine and always absent from portal vein blood when bile was excluded from intestine. Urobilin was found occasionally in jugular blood but only when also in portal blood and always in less amounts. The amount of urobilin in portal blood averaged .008 mg. per cc., and that of the jugular blood .0006 mg. per cc.

The protocol given herewith is of the animal from which portal blood in all three phases was obtained; that is to say, portal blood when there was bile in the intestine, portal blood when there was no bile in the intestine, and portal blood when bile had been returned again to the intestine.

Protocol.

Dog 4, dachshund, 8 kilos.

October 22, 1925. Ether anesthesia. Operation for portal vein sampling and for "altercursive intubation" of common duct.

1st day after operation. Dog active, wound clean.

4th day after operation. Tapped portal vein for 30 cc. Portal blood was negative for bilirubin, positive for urobilin (.0036 mg. per cc.). Jugular blood negative for bilirubin, positive for urobilin (.00054 mg. per cc.). Clamp put on for bile collection.

27th day after operation. Stool and urine negative for urobilin and bilirubin. Bile urobilin-free. Tapped portal vein, 10 cc., negative for bilirubin and urobilin. Jugular vein blood negative for bilirubin and urobilin. Clamp put on for return of bile to intestine.

29th day after operation. Stools contain much urobilin. Urine small amount. Tapped portal vein, 6 cc., with considerable difficulty. Bilirubin negative, urobilin positive (.0018 mg. per cc.). Jugular blood negative for bilirubin, negative for urobilin.

Lymph Absorption.

Absorption by way of the lymph channels was next investigated and lymph obtained by intubating the thoracic duct in the neck was examined for bilirubin and urobilin under various conditions of

intestinal absorption.   In every instance dogs were used, the animals being under ether anesthesia throughout the operation and the period of collection.   Animals that had been fasting, and hence presumably not digesting and absorbing, animals that were liberally fed, and animals with enteric injections of bile and chyme containing bile were examined.   Lymph specimens were tested for bilirubin and urobilin and compared with specimens of jugular vein blood in the same manner as was portal vein blood in the experiments above.

Eleven dogs that had fasted from 1 to 3 days but had abundance of water were examined.   The lymph in five animals contained bilirubin and in six gave no test.   Meanwhile the jugular blood in no instance gave a test for bilirubin.   This same lymph tested for urobilin was positive in ten instances (average .011 mg. per cc.) while the blood was positive for urobilin in nine specimens (average .003 mg. per cc. or about $\frac{1}{4}$ as much).

Twelve dogs liberally fed and watered 2 to 5 hours previous to the experiment were tested in the same manner.   Five of these animals were fed a meal composed of cooked beef lungs, bread, and 200 gm. of lard containing 20 mg. of pure bilirubin dissolved in the lard.   Lard has been found to be a good solvent for bilirubin and in the above proportion is intensely yellow so that it can be diluted many times and still give a positive diazo test.   Furthermore such lard can be emulsified without losing its bilirubin if kept in a neutral or slightly acid medium.   It was thought that bilirubin thus protected from gastric or intestinal digestion might be absorbed into the lacteals after the manner of other fat-soluble dyes.   Seven of the twelve dogs showed tests for bilirubin in lymph and five were negative.   The positive tests were of the same order of magnitude as in the fasted animals, that is not enough to be measured.   At the same time no animal showed bilirubin in jugular blood.   The lymph of these twelve animals in every instance contained urobilin (average .0585 mg. per cc.), while the blood was eleven times positive, averaging .00531 mg. per cc.   From this it can be seen that feeding had increased very materially the amount of urobilin of lymph and blood, but had practically no effect on bilirubin of either.

Six of the fasting animals tested for absorption by way of lymph were injected with bile containing mixtures directly into the duo-

denum, either by a previously established "altercursive intubation" or by syringe and needle into the exposed gut. Two of these animals received bile-stained chyme removed from another dog supposedly at the height of digestion.

This procedure had little or no effect on the bilirubin content of jugular vein blood for whereas all six animals had no bilirubinemia prior to injection only one showed any bilirubinemia after the injection and that one in amounts too small to be measured. Breast lymph and mesenteric lymph (collected in a manner shown below) also showed little or no change, being practically free of bilirubin at all times.

The injection did, however, cause urobilin to increase, and the jugular vein blood of six animals which, before injection, were three negative and three positive, after injection became five positive and one negative. Breast lymph was similarly affected as shown by Table I.

These experiments to investigate the absorption of pigment by way of the lymph (twenty-three in all) showed very clearly that when intestinal absorption was active urobilin was present in lymph in amounts greater than in systemic blood, and furthermore that when the concentration of pigment in the intestine was increased by injection, urobilin of the lymph was also increased.

It must be noted, however, that bilirubin was present in the lymph in twelve out of twenty-three tests, occurring slightly oftener in fed animals and injected animals than in fasting animals as shown by Table I. This does not indicate absorption of pigment, for such amounts of bilirubin might come directly from the liver by way of the liver lymphatics. The anesthesia did not cause bilirubin to appear in lymph for the general tendency was in all cases towards lesser amounts of pigment as the ether was prolonged. The observation that injections of bilirubin into the intestine did not increase the bilirubin content of the lymph argues very strongly that bilirubin of the lymph was not absorbed bilirubin.

To test the matter of the source of this pigment more critically five animals were operated in a manner to expose the lymphatics of the small intestine in the mesentery just as they enter the receptaculum chyli.

TABLE I.

A. *Absorption by Portal Vein. Comparison of Bile Pigment Content of Portal Vein Blood and Jugular Vein Blood in Healthy Animals.*

B. *Comparison of Bile Pigment Content of Breast Lymph and Jugular Vein Blood of Eleven Fasting Dogs.*

C. *Comparison of Pigment Content of Breast Lymph, Mesenteric Lymph, and Jugular Vein Blood.*

| | A | | B | | C | | |
| | Seven healthy animals | | Eleven fasting dogs | | Twelve fed dogs | | |
	Portal vein	Jugular vein	Breast lymph	Jugular vein	Breast lymph	Mesenteric lymph	Jugular vein
Bilirubin positive........	0	0	5	0	7	3	0
Bilirubin negative........	19	19	6	11	5	2	12
Urobilin positive........	9	8	10	9	12	5	11
	(Average .008 mg. per cc.)	(Average .0006 mg. per cc.)	(Average .011 mg. per cc.)	(Average .003 mg. per cc.)	(Average .0585 mg. per cc.)		(Average .0053 mg. per cc.)
Urobilin negative........	*3	**4	1	1	0	0	1

* In these three instances the animal was acholic on account of bile diversion by way of "altercursive intubation."

** In three instances the animal was acholic on account of bile diversion.

D. *Absorption by Way of Lymph. Comparison of Lymph with Jugular Vein Blood before and after Injection of Bile into Intestine.*

| | Six injected dogs | | | | | |
| | Before injection | | | After injection | | |
	Jugular vein	Breast lymph	Mesenteric lymph	Jugular vein	Breast lymph	Mesenteric lymph
Bilirubin positive........	0	2	2	1	3	2
Bilirubin negative........	6	3	0	5	2	0
Urobilin positive........	3	3	2	5	4	2
Urobilin negative........	3	3	0	1	1	0

This was done under ether anesthesia which was continued the entire time of the experiment. The intestines were protected from drying by large sheets of rubber dam and all bleeding avoided as carefully as possible. To make a good exposure the abdominal walls were cut down transversely between heavy wire ligatures and all bleeding points tied or clamped. A large lymph channel was then cannulated with a fine glass tube bent at a convenient angle and with a bulb blown in the stem to hold about 3 cc. When the cannula was in place suction applied by a rubber tube caused the cannula and its bulb to fill with lymph. The full bulb was then emptied with a Wright's pipette and in this manner as much as 30 cc. was collected for comparison with blood from the jugular vein.

Of five such specimens three gave positive diazo tests for bilirubin (the greatest being .02 of the van den Bergh unit). While the blood from jugular vein was negative.

All five contained urobilin and averaged .085 mg. per cc. or about the same order of magnitude found in thoracic duct lymph.

This evidence is very suggestive that small amounts of bilirubin were absorbed, for lymph collected in this manner could come from nowhere save the intestine. There remains, however, the possibility that this small amount of bilirubin could have been formed by the peritoneal membranes of the intestine from hemoglobin free in the belly for it is impossible to say that there was no bleeding and the duration of the experiment—*i.e.*, several hours—might possibly be occasion for bilirubin to be formed. The evidence as to urobilin on the other hand is conclusive that this pigment is absorbed from the intestine into the lymphatics.

COMMENT.

The experiments reported here require little comment by way of explanation and even less in interpretation. The observations on the portal vein blood and the jugular vein blood of the seven normal dogs are conclusive—that bilirubin is not and that urobilin is absorbed from the intestines. The proposition that absorbed pigment is in part or altogether removed by the liver from the blood is in fact a corollary to the proof that urobilin is absorbed at all. Such a function of the liver is further suggested by the fact that portal vein blood—as well as lymph from the intestine—always contains more urobilin than does blood obtained elsewhere—that is to say distal to the liver as located in reference to intestine. It is not vital to this paper to know what

becomes of biliary pigments once they are absorbed from the intestine. It is interesting to note that blood that has been through the liver contains much less of this pigment than do blood and lymph coming direct from the intestine. In none of these experiments was anything observed at variance to the idea that urobilin absorbed from the intestine appears in urine and in bile.

Table I is appended to show in condensed form all the data mentioned in the text as well as many other details omitted above for the sake of brevity. The table is self-explanatory.

The writer is indebted to Dr. P. D. McMaster of The Rockefeller Institute for assistance in planning and executing these experiments.

CONCLUSIONS.

Bilirubin as such is not reabsorbed from the intestine by way of the portal vein in healthy animals. Bilirubin may be absorbed from the intestine by the lymphatics but only in minute amounts.

Urobilin is reabsorbed from the intestine by way of the portal vein and by way of the lymphatics.

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SOME OBSERVATIONS ON THE LYMPH AND BLOOD VESSELS OF THE RABBIT TRACHEA.

WITH PARTICULAR REFERENCE TO THEIR RÔLE IN THE PATHOGENESIS OF EXPERIMENTAL LOBAR PNEUMONIA.

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PLATES 5 AND 6.

Blake and Cecil (1) in 1920 presented evidence to show that in lobar pneumonia the pathological process in the lung parenchyma is secondary to a lymphangitis in the submucosa of the main bronchi near the hilum. This point of view is supported by Winternitz, Smith, and Robinson (2) who, by the injection of India ink into the recently sacrificed rabbit's tracheal submucosa, believed that they had demonstrated an unusually rich lymphatic network which was in communication with the lymphatics at the tracheobronchial junction, these in turn communicating with lymphatics leading to the parenchyma of the lung.

The injection method of Winternitz and his colleagues consisted of the insertion of a fine hypodermic needle beneath the mucosa of the trachea. With very gentle pressure on the plunger a rich plexus of vessels was uniformly outlined by the injected India ink. The photomicrographs which accompany this paper demonstrate a profuse anastomotic network of ink-filled channels in the submucosa of the trachea.

In the course of experiments to determine the routes of absorption of various dyes from the pericardial cavity we attempted to outline the peritracheal lymphatics and to repeat the injections reported by Winternitz, Smith, and Robinson.

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Experimental Methods and Data.

Rabbits were killed, with an occiput blow. The trachea was then bluntly freed from the surrounding tissues. An incision was made anteriorly in the midline of sufficient length to allow for the injection procedure. A fine 27 gauge platinum hypodermic needle attached to a 2 cc. syringe containing either full or half strength Higgins' India ink was carefully inserted beneath the tracheal epithelium. With gentle pressure on the plunger a rich plexus of ink-filled vessels immediately sprang into view. It was important to avoid holding the trachea on the stretch for this impeded the filling of the channels. With proper pressure and a few changes in the site of injection the entire submucosa of the trachea was outlined by the short, stubby, richly anastomosing ink-filled channels (Fig. 1).

In the midst of one of these injection experiments it was noted that some of the dye was filling a vessel about 1 mm. in diameter lying in the supporting tissue, parallel to and to the left of the trachea. Another vessel filled with ink, smaller than the first, lay in the loose tissue to the right of the trachea. With the aid of the dissecting microscope the smaller of the two channels was traced into the peritracheal tissue, whereas the larger was found to run along the entire length of the trachea, receiving branches from that organ and emptying into the superior vena cava just before this vessel entered the right auricle. Particles of ink were found in the superior vena cava and the right auricle.

Another preparation was made in the following fashion. The rabbit was killed and the trachea slightly freed from its surrounding structures. The vein parallel and to the left of the trachea, referred to above, was identified, tied towards the heart, and injected towards its origin. In a few moments the rich plexus of vessels in the tracheal submucosa became partially filled with the India ink and many veins from the angle of the jaw to the clavicle became stained with the dye.

In a third experiment the rabbit was sacrificed by an occiput blow, the entire trachea removed and slit in the anterior midline. The preparation was then inspected under the dissecting microscope. A plexus of short, stubby, richly anastomotic blood vessels ramified in the submucosa. These vessels were identical with the network which had been injected with ink in the previous experiment and were similar to the channels described by Winternitz and his coworkers as lymphatics.

The hypodermic needle was then inserted into the submucosa and an attempt was made to demonstrate the presence of lymphatics as distinct from the blood vessels. With the aid of a dissecting microscope it could easily be seen that the ink granules entered the meshwork of vessels and pushed the blood cells before them. Repeated attempts to inject lymphatics always met with the same results although five rabbits were used and each trachea subjected to many injections under the microscope.

Pieces of normal rabbit trachea and of tracheæ injected with India ink were fixed in Zenker's and stained with Giemsa.

Examination of the normal trachea demonstrated that the vascular arrangement is of an unusual order (Fig. 2). An extremely rich supply of very thin walled anastomosing blood vessels ramifies in the submucosa. In some sections a single vessel extends about one-fourth the circumference of the trachea. Many little outpocketings designating the junction of tributaries are observed in these larger channels. The walls of these vessels consist of a single layer of endothelial cells with a very delicate connective tissue stroma which is best brought out with Mallory's aniline blue stain. If these thin walled vessels are not filled with blood it is very difficult to distinguish them from lymphatics. It is possible, however, to identify lymphatics and the submucosa but they are of very small calibre, relatively few in number when compared with the blood vessels, and the supporting connective tissue for the latter is slightly more prominent than that for the lymphatics.

The large bronchi contain the same arrangement of blood vessels.

Sections through the trachea which have been injected with India ink present one of two pictures. In the one the red and white cells of the vessels have been completely replaced by ink particles and photomicrographs of these regions resemble those described by Winternitz, and his collaborators, as being ink-filled lymph channels. In the other group of sections, ink granules and blood cells may be seen mingled in the same vessel (Fig. 3).

The human trachea[1] was examined to ascertain whether there existed in the submucosa a vascular supply comparable to that

[1] We wish to thank Dr. Charles Connor of the Pathology Department of the Peter Bent Brigham Hospital for his aid in obtaining this material.

found in the rabbit. The thickness of the mucosa made it impossible to study the vascular channels under the dissecting microscope. Nevertheless, strips of the normal human trachea were subjected to the technique of a blind stab and injection into the submucosa. The results derived from this procedure were unexpectedly satisfactory. Grossly it could be readily seen that two independent types of vessel plexuses existed. Sometimes the one type and sometimes the other was injected. One plexus was finer than the other. Microscopic examination of sections prepared from the injected areas showed these channels to be lymphatics which are smaller and less numerous, than the blood vessels (Fig. 4).

The second type of plexus injected is made up of small blood vessels. These vascular structures bear no resemblance to the rich blood supply found in the rabbit trachea.

SUMMARY AND CONCLUSIONS.

1. Winternitz, Smith, and Robinson have offered as experimental evidence in support of the lymphatic path of infection in lobar pneumonia, a series of injection experiments of the rabbit's tracheal submucosa.

What these investigators supposed to be lymphatics are shown by us to be an unusual type of venous supply consisting of short, wide, and richly anastomosing vascular channels.

2. There is little homology between the structure and distribution of the lymph and blood vessels in the human and rabbit trachea.

3. Injections of pneumococci by the tracheal route to produce lobar pneumonia in the rabbit include an intravenous administration of these organisms when the mucosa is pierced or injured.

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EXPLANATION OF PLATES.[2]

PLATE 5.

FIG. 1. Blood vessels of the submucosa of the rabbit's trachea injected with India ink. Preparation cleared by the method of Spalteholz.

FIG. 2. Blood vessels in the submucosa of the rabbit trachea.

PLATE 6.

FIG. 3. Blood vessels in the submucosa of the rabbit trachea partially filled with India ink.

FIG. 4. Submucosa of the human trachea. The lymphatics are filled with India ink.

[2] We are indebted to Dr. S. B. Wolbach for the photomicrographs.

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PLATE 5.
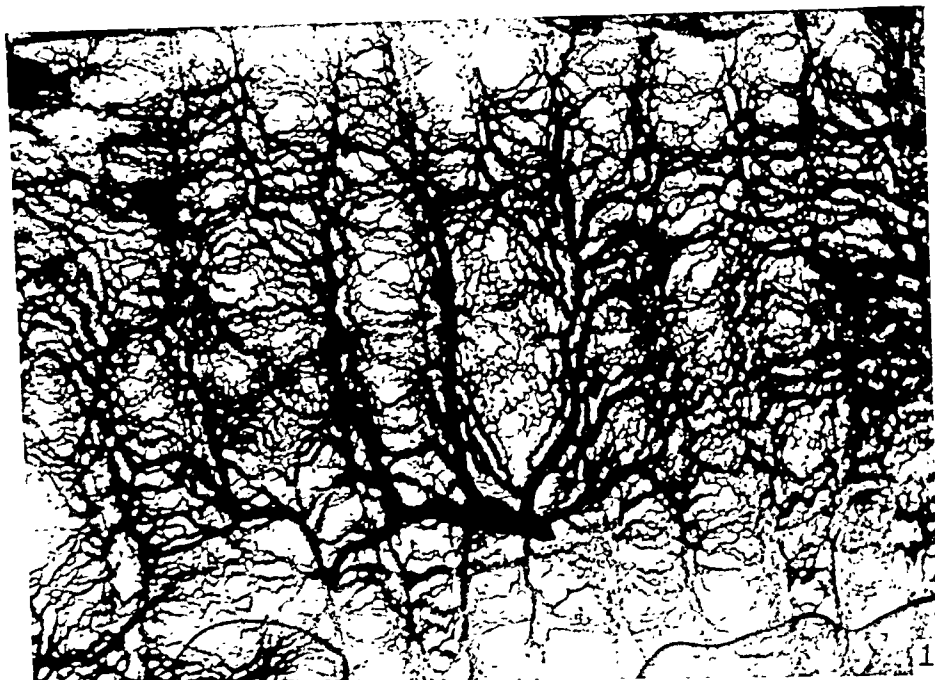
(Seegal and Seegal: Lymph and blood vessels of rabbit trachea.)
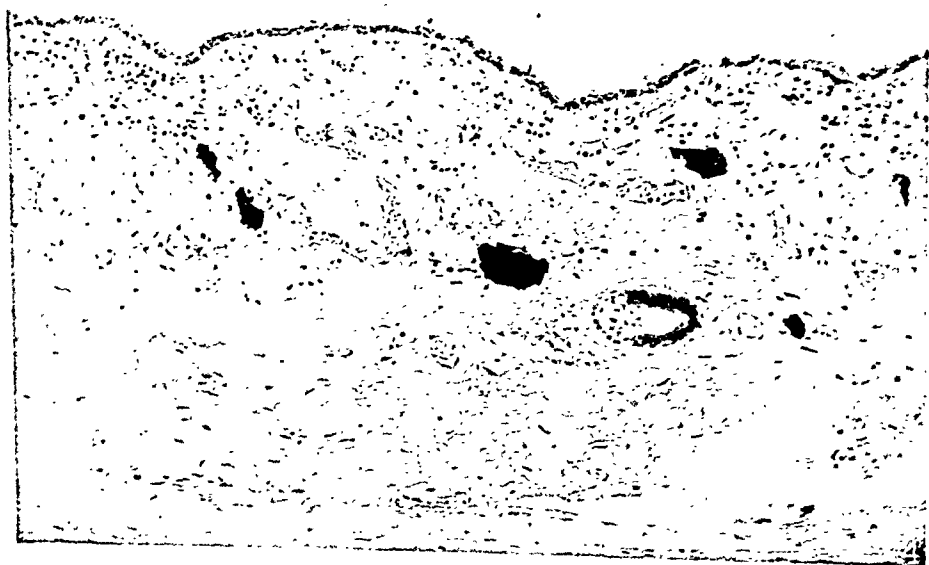
(Seegal and Seegal: Lymph and blood vessels of rabbit trachea.)

EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS.

By DAVID PERLA, M.D.

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The pioneer studies in experimental epidemiology made with mouse typhoid at The Rockefeller Institute by Flexner (1), Amoss (2), Lynch (3) and the extensive experiments of Webster (4) and Pritchett (5), which together with the work of Topley (6) in England initiated the new field of experimental epidemiology, have suggested studies along similar lines in the experimental epidemiology of tuberculosis. At the suggestion of Professor Eugene L. Opie, I have undertaken some preliminary experiments in artificially induced epidemics of tuberculosis in guinea pigs.

Studies with tuberculosis present numerous difficulties not encountered in a more rapidly promulgated disease such as mouse typhoid. The duration of the disease, the nature of the infection, the difficulty of spontaneous transmission from animal to animal and the chronicity of the resulting infection make experimental epidemiological studies in tuberculosis a matter of years. Greater hazards than usually occur in animal experimentation are involved in maintaining large groups of guinea pigs alive for months in the same cage.

In the course of the preceding year it was noted in this laboratory that several instances of spontaneous tuberculosis occurred in guinea pigs and rabbits which either had been injected with some innocuous material in the course of other experiments or had been caged merely in the same room with tuberculous animals. These are reported in detail later in this paper. It seemed essential to determine under experimentally controlled conditions the frequency of the occurrence of spontaneous tuberculosis and the factors in its spread. The results of these preliminary studies are presented in this paper.

Reports of spontaneous tuberculosis in mammals other than man, cattle and swine are numerous. Calmette (7) in a review of the literature of spontaneous

tuberculosis in mammals states that it is very rare in the horse, ass, goat, sheep, cat, rabbit, guinea pig and rat but may occur in these animals if they are exposed for long periods to tuberculous cattle or man. It is well known that tuberculosis is very frequent in captive monkeys. Both human and bovine strains have been isolated. Animals in the wild state are said never to contract tuberculosis. While examining ground squirrels captured in the wild state, for the plague, five instances of natural tuberculous infection of the bovine type were observed by McCoy and Chapin (8).

In the Philadelphia Zoological Garden, Fox (9) found tuberculosis in nearly all the vertebrates examined. In a series of 1860 autopsies on Mammalia, 14.7 per cent died from naturally acquired tuberculosis. Of all primates autopsied, 48.5 per cent were tuberculous; of Lemures, 26.7 per cent; of Carnivora, 3.5 per cent; of the Rodentia, 2 5 per cent; of the Ungulata, 9.6 per cent; of the Proboscidea, 66 per cent (2 of 3 instances). A study of the pathological types observed reveals the frequency of the "nodular and caseous forms." No fibroid tuberculosis approaching the quiescent type as seen in man and rarely in domestic animals was encountered. Calcareous deposits were extremely rare. The most susceptible tissue of wild animals in confinement is the lung. The probable origin of the infection in all orders of the Mammalia of this series was bronchiogenic in about half of the instances and intestinal and lymphatic in the other half.[1]

Rabbits and guinea pigs exposed in the same room with tuberculous animals for a longer period than 4 months, Koch (10) found not infrequently developed spontaneous tuberculosis. This significant observation made 40 years ago has not been adequately appreciated by workers in tuberculosis. The occurrence of natural infection was in direct proportion to the number of tuberculous animals present in the room. According to Koch, the type of disease in spontaneous tuberculosis in these animals is characteristic. The lungs contain large caseating areas with occasional cavitation and the hilum nodes are enormously enlarged and caseous. This striking picture in sharp contrast with the pathology of induced infections is evidence of the respiratory route of infection. Koch emphatically states that if rabbits or guinea pigs have remained 3 or more months in the same room with tuberculous animals, the experiments in which these animals are used cannot be relied upon, unless spontaneous tuberculosis can be excluded. Isolated instances of spontaneous tuberculosis in rabbits were reported by Strauss (11) and Guérin (12) and an epizootic of spontaneous tuberculosis of the bovine type on a rabbit farm was studied by Rothe (13). He observed that tuberculosis was readily contracted by normal animals when directly exposed to infected cage mates. If, however, a normal animal merely occupied the cage where an infected animal had lived, it did not contract tuberculosis. He fails to describe the pathological anatomy of the disease. Within 4 years, Couland (14) observed seventeen instances of spontaneous tuberculosis in rabbits. The lesions were primarily in the lungs.

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[1] A complete review by Eber of the recent literature of tuberculosis in the lower animals may be found in *Ergebn. allg. Path. u. path. Anat.*, 1917, xviii, pt. 2, 1.

One group showed large masses with caseation; another showed discrete tuberculous lesions in the lungs, kidneys and liver. Three of the four offspring of a rabbit that had been inoculated intravenously 8 days prior to parturition developed tuberculosis of the human infantile type (15). On the basis of feeding experiments, in an attempt to determine the pathogenesis of the naturally acquired infection in these animals, Couland concluded that intestinal and mesenteric lesions developed in rabbits that had been fed massive doses of bacilli. With smaller doses, pulmonary lesions similar to those found in spontaneously infected rabbits were observed. The development of these lesions took several months. It is probable that the tubercle bacilli after ingestion penetrated past the epiglottis into the respiratory tract.

In studying the route of spontaneously acquired tuberculosis in a small series of guinea pigs exposed to phthisical patients for several days and then isolated for 30 days, Bartel and Spieler (16) examined sections of the base of the tongue, pharyngeal wall, bronchial, cervical and mesenteric lymph nodes, the trachea and the lungs. The pharyngeal wall showed a microscopic tuberculous lesion in one instance, the lungs and tracheobronchial lymph nodes, occasionally, but the mesenteric lymph nodes most frequently. In exposing guinea pigs to a spray of emulsions of tubercle bacilli during 5 to 10 minutes and again examining the various tissues, Bartel and Neumann (17) found the tracheobronchial lymph nodes most frequently involved although mesenteric and cervical lymph nodes were occasionally affected. Lesions in the tonsils were not present in either the sprayed or exposed animals. The experiments, however, are inconclusive.

Recently, Remlinger (18) confined healthy guinea pigs in the same cage with others having open tuberculous ulcers; 14 per cent of his normal contact animals contracted tuberculosis. Of the normal guinea pigs exposed to infected cage mates that were free from open lesions, 9 per cent developed tuberculosis. Normal guinea pigs confined in cages in which others had recently died of generalized tuberculosis remained well. Of 52 guinea pigs fed for 1 year exclusively on barley soiled with the fecal material of tuberculous guinea pigs, not one developed tuberculosis. Remlinger does not specify the duration of infection in the tuberculous animals at the time feces were obtained nor the quantity of bacilli used in infecting these animals. Sewall and Lurie (19) observed an epizootic of spontaneous tuberculosis in guinea pigs that had been exposed to polluted air and confined for 7 to 18 months in the laboratory of a sanitorium for tuberculous patients. The exact pathology of the lesions is not described.

Spontaneous Tuberculosis in Rabbits and Guinea Pigs.

The occurrence of spontaneous tuberculosis in a rabbit or guinea pig in a laboratory where tubercle bacilli are constantly used for experimental purposes may be due either to naturally acquired infection or, where non-tuberculous material has been injected into

the animal, to wound contamination. Several instances of tuberculosis due to wound contamination have developed in our laboratory in the past year. The discovery of tubercle bacilli in the lesion at the site of inoculation indicates that the infection has entered through the wound. The following instances of spontaneous tuberculosis and tuberculosis due to wound contamination occurred in the general animal room of the Henry Phipps Institute. In these instances a diagnosis of tuberculosis was made on the basis of the pathological findings at autopsy and the presence of acid-fast bacilli in smears made from the involved organs. Animal inoculations were not made.

Rabbit 1[2] was injected with horse serum subcutaneously in the back and died 120 days later. Autopsy—At the site of inoculation in the subcutaneous tissue of the back is a small abscess filled with caseous material which contains numerous bacilli with acid-fast characters. The lungs are studded with grey spots 1 mm. in diameter and several caseous nodules from 2 to 4 mm. in diameter are present. In the right upper lobe close to the hilum, there is a thick walled cavity 1.5 cm. in diameter filled with cheesy yellow material. This infection is probably due to wound contamination.

Rabbit 2 was repeatedly injected with crystalline egg albumin. It died 155 days following the first injection. Autopsy—Tuberculous nodules 8 mm. in diameter are present in both upper lobes of the lungs. The hilum lymph nodes are not enlarged. The spleen and liver are slightly enlarged. There is no lesion at the site of inoculation. This is possibly an instance of bronchiogenic spontaneously acquired infection.

Rabbit 3 died 185 days following the injection of horse serum. Autopsy revealed one large caseous nodule in the left lung close to the hilum. There was no other sign of tuberculosis. This is another instance of spontaneously contracted tuberculosis introduced through the respiratory tract.

Guinea Pig 1 was injected with 12 cc. of horse serum and died 116 days later. Autopsy—At the site of inoculation is a caseous nodule 4 mm. in diameter. The omentum is thickened and studded with yellow caseous spots and fibrous tissue The spleen is from fifteen to twenty times the normal size and is reddish grey in spots with numerous small caseous areas throughout. The liver is much enlarged and contains numerous yellow areas, 8 mm. in diameter. The splenic lymph node is 1.5 cm. in diameter and is caseous on section. The lungs are studded with grey-yellow spots 3 mm. in diameter. This is obviously an infection due to the accidental introduction of tubercle bacilli at the site of the serum injection.

Guinea Pig 2 was injected with .5 cc. of horse serum intravenously and died 135 days later. Autopsy—The submaxillary and deep cervical lymph nodes are en-

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[2] For the purpose of citation animals are numbered consecutively.

larged, firm, from 3 to 4 mm. in diameter and on section show caseous material. The hilum lymph nodes measure 4 mm. in diameter, are matted together and filled with cheesy material. The lungs are studded with confluent grey spots. The spleen is six times the normal size and contains numerous large caseous spots. The liver is much enlarged and contains small yellow, necrotic and irregular patches. One iliac lymph node is enlarged and caseous. This was an instance of spontaneous tuberculosis probably introduced through the pharynx, spreading by lymphatic channels and finally reaching the respiratory tract with general dissemination.

All the animals reported above had been kept in separate cages in the general animal room and tuberculosis was incidentally discovered during the course of experiments not concerned with tuberculosis.

To test the incidence of spontaneous tuberculosis in the general animal room, four cages each containing four guinea pigs were placed in various parts of the animal room. One cage was placed in the northeast corner among rabbits treated with horse serum in the course of other experiments; one among cages containing guinea pigs injected with suspected tuberculous material; another in the southeast corner among a similar group of animals; and a fourth among tuberculous rabbits. Two instances of spontaneous tuberculosis developed in these animals and both were in the group that had been placed among the cages containing tuberculous rabbits.

Guinea Pig 3 lived 132 days after exposure and was killed to obtain serum. Autopsy—The spleen is slightly enlarged and nodular. The liver shows a few yellow spots. In the center of the left lung there is a thick walled cavity containing cheesy necrotic material. A smear taken from the wall of the cavity shows acid-fast organisms. The tracheobronchial lymph nodes measure 2 cm. in diameter, are matted together and are firm. On section they contain caseous material. There is a complicating adhesive pleurisy and chronic pneumonia of the left lung. The portal of entry of the infection in this instance was the respiratory tract.

Guinea Pig 4 died after 278 days. Autopsy—There is no local lesion. The iliac and lumbar lymph nodes are slightly enlarged. The spleen is five times the normal size, friable and contains several caseous areas. The liver contains a few small patches of yellow necrotic tissue from 2 to 3 mm. in diameter. The lungs are studded throughout with grey-white tubercles, with occasional opaque yellow central spots. The tracheobronchial lymph nodes are large, firm and caseous on section.

The portal of entry in both instances was the respiratory tract. It is apparent that bronchiogenic infection is possible among guinea

pigs exposed to tuberculous animals although isolated in separate cages.

Experimental Studies of Spontaneous Tuberculosis in Guinea Pigs.

The following experiments were undertaken to determine how frequently spontaneous tuberculosis occurs under controlled conditions in guinea pigs exposed to tuberculous guinea pigs. Observation was planned to determine: first, the effect of varying the degree of exposure; second, the effect of crowding on the incidence of spontaneous tuberculosis; third, the pathogenesis and pathology of spontaneous tuberculosis acquired by direct exposure to tuberculous animals with "closed" and "open" lesions.

These tests were carried out in a separate animal room in a separate building away from all possible source of outside contamination and exposure other than that introduced directly into the experiment. The room measured 15 × 8 × 10 feet. There was one window 5 × 3 feet. The temperature was kept nearly constant throughout the experiment. At the beginning of August, however, the constant temperature could not be maintained and since deaths from causes other than tuberculosis were numerous, the experiments were brought to a close, 7½ months after their beginning. About 460 guinea pigs were kept under observation. When there was any doubt about the character of the lesions which were found, sections were made and animal inoculations were used to confirm the diagnosis. The diagnosis of every case of spontaneous tuberculosis was confirmed by smears prepared from the organs involved and stained for acid-fast bacilli. Care was exercised to exclude the possibility that guinea pigs had been exposed to tuberculosis prior to their introduction into the experiments. The normal animals were obtained from The Rockefeller Institute for Medical Research at Princeton. The number of normal and infected animals was kept constant throughout the experiments by replacing the pigs as they died. The animals were fed the routine diet of oats, moistened bread and lettuce leaves. The attendant who cared for these guinea pigs was not permitted to handle animals in the general animal room. Workers from the general animal room were excluded and special gowns were worn by those entering the room.

The infecting organism was originally isolated from the lungs of a patient with pulmonary tuberculosis. It had been grown on glycerol agar in this laboratory during 2 years. Glycerol agar transplants 3 weeks old were used in inoculating the guinea pigs. The strain was highly virulent.

The Relation of Spontaneous Tuberculosis to Intensity of Exposure.

The attempt was made to determine whether spontaneous tuberculosis occurs in guinea pigs exposed to cage mates infected intra-

peritoneally with virulent tubercle bacilli; to determine the relation of incidence to intensity of exposure and to compare this with incidence of spontaneous tuberculosis in guinea pigs exposed to guinea pigs with open tuberculous lesions.

Experiment 1.—60 guinea pigs of the same weight and age were divided into five series of twelve each and placed in five large cages arranged in vertical series. Each cage measured 39 inches long by 13 inches high by 14 inches deep. The top and bottom cages held twelve normal control pigs. In Cage 2, three animals were infected with .001 mg. P-15-B, a virulent culture of human tubercle bacilli; nine were normal cage mates. In Cage 3, six were infected and six were normal. In Cage 4, nine were infected and three were normal. The number of infected and normal animals was kept constant by continual replacement as the deaths occurred. After the first replacement, .00001 mg. injected intraperitoneally was used to infect the guinea pigs. The average life of a guinea pig injected with .001 mg. intraperitoneally was from 6 to 8 weeks, with .00001 mg. from 2 to 3 months. In some cages, as many as five successive replacements of infected animals were necessary. In infecting the animals, intraperitoneally, every precaution was taken to avoid contamination of the subcutaneous tissue of the skin so that in no instance did an open lesion develop. All animals were killed at the end of 7½ months.
Results.

In the control cages, there were no instances of spontaneous infection.

Cage 2.—There was one instance of spontaneous tuberculosis. Guinea Pig 4 was killed after 225 days. Autopsy—A mesenteric lymph node is slightly enlarged and contains a few yellow caseous spots. The spleen is three times the normal size. The liver contains a few yellow spots.

Cage 3.—There was no instance of spontaneous tuberculosis. Only three of the normal animals, however, survived the entire period.

Cage 4.—Of the three normal animals, two were found to have spontaneous tuberculosis at autopsy.

Guinea Pig 5 died after 127 days. Autopsy—The mesenteric lymph node measures .5 cm., is firm and on section presents several very small caseous areas. The spleen is four times the normal size and is nodular with one or two yellow-grey caseous spots.

Guinea Pig 6 was killed after 225 days. Autopsy—A mesenteric lymph node measuring .5 cm. in diameter is very firm and fibrous and on section there are within its substance a few small caseous spots. The spleen is five times the normal size, studded with greyish spots, 2 mm. in diameter. The liver shows a few yellow areas.

The incidence of spontaneous tuberculosis in this cage was 66 per cent of the original three animals, but 100 per cent of those that survived the experimental period acquired tuberculosis.

From this experiment, it is obvious that spontaneous tuberculosis occurs among guinea pigs directly exposed to cage mates infected intraperitoneally with human virulent tubercle bacilli. Of a total of thirteen normal animals which survived the entire period of the experiment and which were directly exposed to animals infected intraperitoneally with human virulent tubercle bacilli, 22 per cent acquired tuberculosis. The portal of entry in all of these instances was the intestinal tract and the mesenteric lymph nodes. The greatest incidence occurred in the cage where the intensity of the exposure was greatest. A greater number of instances of spontaneous tuberculosis would doubtless have occurred had the experiment continued longer, since infection occurred in most instances in this and the following experiments in animals exposed to infection during the entire experimental period.

The source of infection in this experiment was probably through fecal contamination of the food. Careful studies were made in attempting to estimate the quantity of virulent tubercle bacilli eliminated in the excreta of infected guinea pigs. The results of these studies will be reported in a separate paper. It was found that guinea pigs infected intraperitoneally as well as subcutaneously eject viable tubercle bacilli in their feces during the last few weeks of their lives. In some instances animals injected intraperitoneally eliminated tubercle bacilli during the 1st week of the infection. When it is considered that during the period when tubercle bacilli appear in the feces, approximately one-sixtieth the quantity of feces excreted by one infected guinea pig during 24 hours is capable of producing tuberculosis when injected subcutaneously into a guinea pig, a rough estimate of the quantity of infected material to which the normal guinea pigs were exposed may be obtained. Although the cages were cleaned three times a week, there was a constant source of contamination present from the feces.

Experiment 2.—Five small cages, each 14 × 17 × 14 inches were arranged in vertical series and thirty guinea pigs divided into five groups of six were placed in the cages. The top and bottom cages each held six normal guinea pigs. In Cage 2 were four normal guinea pigs with two cage mates infected subcutaneously with .01 mg. of P-15-B, the same virulent human strain used in the previous experiment. In Cage 3, three normal and three infected animals were placed; in Cage 4, two

normal and four infected. With the quantity of bacilli used local abscess formation with ulceration occurred in many cases during a period from the 2nd to about the 5th week. Very frequently no ulceration resulted. As the infected animals died, they were replaced by other infected guinea pigs. In many instances, replacements were made four times in the course of the experiment. At the end of 7½ months the experiment was brought to a close as in the first experiment. The animals were kept in the same isolated animal room as those of Experiment 1.

Results.

No instances of acquired infection occurred in the control cages.

In Cage 2, there were two instances of spontaneous tuberculosis.

Guinea Pig 7 died after 222 days. Autopsy—A mesenteric lymph node is 1 cm. in diameter, dense and caseous on section. There are a few yellow spots on the serosal surface of the ileum but no definite ulcerations in the intestinal tract. The spleen is three times the normal size, red, with scattered greyish spots. The liver is much enlarged. The right lobe contains several large areas from 3 to 8 mm. in width, which are yellow and caseous on section.

Guinea Pig 8 was killed after 226 days. Autopsy—A fibrous mesenteric lymph node measures .5 cm. in diameter. The spleen is nodular and three times the normal size. On its surface are a few greyish caseous spots. The liver shows a few minute yellow spots.

Of four normal animals, two or 50 per cent acquired tuberculosis. All four animals had survived about the same length of time.

Cage 4.—One animal developed spontaneous tuberculosis.

Guinea Pig 9 died after 117 days. Autopsy—The left submental lymph node measures 4 mm. across, is firm and on section shows several caseous areas. The deep cervical lymph nodes are matted together in a firm mass measuring 1.5 cm. in diameter and containing several areas of caseation. A mesenteric lymph node measures 5 mm. and is fibrocaseous on section. The spleen and liver are moderately involved.

In Cages 4 and 5 there were no instances of spontaneous infection.

In this experiment, of the eight normal guinea pigs originally placed in the cages which were directly exposed to animals with open lesions during 7 months, three or 38 per cent developed tuberculosis. One died of an acquired tuberculosis 117 days after the beginning of the experiment.

In contrast with the first experiment, the incidence in the second experiment is higher. According to Römer (20) every guinea pig directly exposed during 1 year to infected cage mates with open ulcerations acquires tuberculosis. In his classic experiments on reinfection, his pigs were infected with 5 mg. of bovine tubercle bacilli injected subcutaneously into both thighs. The infecting dose was enormous and the resultant ulcers consequently offered abundant

opportunity for infection to normal cage mates. In our experiment much smaller infecting doses were used and ulcerations were obtained in considerably less than half of the instances. The tendency of guinea pigs to lick the open ulcerations of their cage mates is, of course, an obvious means of transmission of the infection.

The pathogenesis of spontaneous tuberculosis acquired by guinea pigs exposed directly to cage mates having open ulcerations is clear. It is noteworthy that infection *via* the intestinal tract with involvement of the mesenteric lymph nodes not infrequently is accompanied by massive infection of the submental and cervical lymph nodes. This association of the mesenteric and cervical lymph node tuberculosis is significant and is probably analogous to childhood bovine tuberculosis in which these lymphatic structures are primarily involved. Massive involvement of cervical lymph nodes was observed in instances of spontaneous tuberculosis incidentally reported in Römer's work.

The Effect of Crowding on the Incidence of Spontaneous Tuberculosis in Guinea Pigs.

The attempt was made to determine the relation of incidence to crowding by increasing the number of normal animals exposed and keeping the source of infection constant.

Experiment 3.—Four large cages, 40 × 14 × 17 inches, were arranged in vertical series in the animal room containing the other experimental cages. In Cage 1 were placed twelve guinea pigs, six normal and six infected intraperitoneally with .001 mg. of a 3 week glycerol agar culture of a virulent human strain of tubercle bacilli, P-15-B, the same strain used in the other experiments. In the later period of the experiment, infected animals which died were replaced by others in which .00001 mg. was used as the infecting dose. The average duration of life with .001 mg. was 6 weeks; with .00001 mg., 2 or 3 months; with .000001 mg., 3 or 4 months. In Cage 2, were placed eighteen guinea pigs, six infected with twelve normal cage mates. In Cage 3 were twenty-four guinea pigs, six infected with eighteen normal cage mates. In the lowermost cage were thirty guinea pigs; six were infected and twenty-four were normal. The control cages of Experiment 1 served as controls for this experiment as well. The cage used in the present experiment houses twelve pigs without evident crowding. The duration of the experiment was 7 months.

Results.

Cage 1.—There were no instances of spontaneous tuberculosis; four of the original six animals survived during the entire experimental period.

Cage 2.—There were two instances of spontaneous tuberculosis.

Guinea Pig 10 was killed after 210 days. Autopsy—A mesenteric lymph node 1 cm. in diameter is densely fibrous. The spleen is four times the normal size and contains numerous greyish pink spots slightly elevated above the surface, the spots varying from 1 to 3 mm. in diameter.

Guinea Pig 11 at the end of 94 days of exposure gave birth to one animal. It was removed from the cage during the remainder of the experiment and placed in a separate cage with its offspring. 68 days later, it again gave birth to a litter of three. It died after 192 days. Autopsy—A mesenteric node is much enlarged measuring 1.5 cm., is firm on section and contains two small caseous spots. The spleen is five times the normal size; it has a granular surface and contains numerous caseous spots. The liver is slightly enlarged and shows a few scattered small necrotic areas.

Of the twelve normal guinea pigs originally placed in the cage, seven survived during the entire experimental period. The incidence of spontaneous tuberculosis was 29 per cent. The total number of animals used in this cage was forty-seven.

Cage 3.—Of eighteen normal animals originally included in the experiment, twelve survived during the entire period and nine developed spontaneous tuberculosis. In this cage, however, two animals both tuberculous were found partly eaten after death. These animals died 4 months after the beginning of the experiment.

Guinea Pig 13 was killed after 212 days. Autopsy—The mesenteric nodes are enormously enlarged, matted together in a mass 2 cm. long by 1 cm. in diameter, densely fibrous and contain a few areas of caseation. The spleen is three times the normal size; its surface is granular with minute, fibrous, grey-white spots. The liver shows a few small yellow spots. The submental node on the right and the deep cervical node on both sides measure 8 mm. in diameter. They are firm, fibrous and contain minute caseous areas.

Guinea Pig 14 died after 152 days. Autopsy—The peritoneum is coated with a thick greenish white fibrinopurulent exudate. The spleen and liver are coated with exudate but otherwise normal. Buried among the adhesions in the mesentery is a large firm lymph node 1.5 cm. by 1 cm. which cuts with difficulty and on section shows two areas, 3 and 6 mm. in diameter, of caseous material. There is no other evidence of tuberculosis in the body. There is, however, chronic purulent pleurisy and pneumonia and chronic pericarditis.

Guinea Pig 15 was killed after 212 days. Autopsy—The mesenteric nodes are matted together in a mass 2 cm. in diameter. On section the mass is yellowish in

color and densely fibrous. The spleen is three times the normal size and is nodular. There are one or two yellow spots in the liver.

Guinea Pig 16 was killed after 212 days. The mesenteric nodes are matted together in a mass 2 cm. in diameter. On section the mass is yellowish in color and densely fibrous. The spleen is three times the normal size and is nodular. There are one or two yellow spots in the liver.

Guinea Pig 17 died after 132 days. Autopsy—Almost the entire mesentery is occupied by a large mass 1.5 cm. in diameter, irregular in shape and moderately firm. On section it contains a large area of caseation which is readily removed leaving a thick walled cavity. There is a mass of caseous nodes about 1 cm. in diameter in the region of the upper end of the jejunum and another in the region of the cecum. The spleen is slightly enlarged and is nodular.

Guinea Pig 18 died after 197 days. Autopsy—The mesenteric lymph nodes are matted together in a firm mass measuring 1.5 cm. in diameter, and containing a few caseous spots. In the ileum are four or five deep ulcers each measuring 3 mm. in diameter. The floor of the craters is necrotic. The surrounding tissue is deeply congested. The spleen is nodular and measures four times the normal size. The liver contains numerous yellow spots. The lungs contain scattered grey spots measuring from 1 to 3 mm. in diameter with caseous centers. The hilum nodes are slightly enlarged and contain a few caseous spots. A smear taken from the floor of the ulcer contains acid-fast organisms.

Guinea Pig 19 died after 121 days. Autopsy—The mesenteric nodes are matted together in a firm mass 1.5 cm. in diameter and on section contain several small areas of caseation surrounded by dense fibrous tissue. The spleen is four times the normal size and nodular. The liver contains numerous yellow spots. The iliac, lumbar and hepatic nodes are slightly enlarged and contain minute caseous spots. The ileum contains on its mucosal surface several raised hemorrhagic circular ragged ulcers 4 mm. in diameter which extend to the serosa. On the serosal surface directly over the ulcers are small caseous spots 1 mm. in diameter. The lungs contain scattered grey spots 1 mm. in diameter. Both submental nodes measure 1.5 cm. in diameter, are firm, dense, cut with difficulty and on section contain several caseous areas in their centers. The deep cervical nodes are slightly enlarged.

Guinea Pig 20 died after 139 days. Autopsy—Two mesenteric nodes are much enlarged and contain on section small areas of caseation. The liver contains numerous yellow spots. The spleen is slightly enlarged; its surface is granular. The lungs contain scattered grey spots.

In Cage 3, there were nine instances of spontaneous tuberculosis. The portal of entry in all of these was apparently the intestinal route. Several showed involvement of the cervical lymph nodes. Two had tuberculous ulcerations in the ileum, which is a most unusual finding in experimental tuberculosis. Of the twelve normal animals that

survived the experimental period, seven developed tuberculosis. About 60 per cent of the normal guinea pigs which were originally placed in the cage and which survived the experimental period, developed tuberculosis. One guinea pig developed a fatal tuberculosis within 121 days.

Cage 4.—There were four instances of spontaneous tuberculosis in this cage. Of twenty-four normal animals originally placed in the cage, only eleven survived the experimental period.

Guinea Pig 21 died after 80 days. Autopsy—There is a disseminated tuberculosis involving the omentum, liver, spleen and lungs. The hilum nodes are matted together in a large firm mass measuring 1.7 cm. in diameter and on section contain areas of caseation surrounded by dense fibrous tissue. All other nodes appear to be normal. The portal of entry in this instance was probably the respiratory tract.

Guinea Pig 22 was killed after 212 days. A mesenteric node measuring .5 cm. in diameter is dense and on section entirely fibrous. The spleen is three times the normal size and is nodular with minute caseous spots. In all probability the mesenteric lesion had healed.

Guinea Pig 23 was killed after 212 days. Autopsy—A mesenteric node measuring 6 mm. in diameter is densely fibrous on section. The spleen is four times the normal size, is nodular and contains two or three small yellowish spots. The liver contains a single yellow area 2 mm. in diameter. The left submental node measures 4 mm. in diameter and is firm and fibrous on section.

Guinea Pig 24 was killed after 149 days. Two mesenteric lymph nodes are enlarged, each measuring 4 mm. in diameter. On section, they are fibrous and contain one or two minute areas of caseation. The spleen is three times the normal size; its surface is granular and it contains throughout numerous minute grey-white spots. The liver contains two yellow spots, 2 mm. in diameter.

About 36 per cent of the normal animals surviving the experimental period developed spontaneous tuberculosis. In this cage, in three of the four animals that developed tuberculosis, the digestive tract was the portal of entry, but in one instance the infection was possibly bronchiogenic.

44 per cent of the normal animals originally placed in the cages in this experiment developed spontaneous tuberculosis. The results are indicated in Table I. As may be gathered from the table the greatest incidence did not occur in the most crowded cage. Crowding, however, is doubtless a factor in the spread of spontaneous tuberculosis. If in any group of confined guinea pigs exposed to a given intensity

of infection a definite percentage acquire spontaneous tuberculosis, a greater number of instances will appear in the larger groups of animals. This increase in the number of infected animals in the cage results in an increase in the intensity of exposure for other members of the group.

The experimental period, however, was too short to determine any definite relation between crowding and the incidence of spontaneous infection. Although the total incidence of spontaneous disease in this series is greater than the incidence in the other series where crowding was not a factor, the conditions, notably the intensity of the infection, present in the various cages were different.

TABLE I.

Crowding and the Incidence of Spontaneous Tuberculosis.

Cages	No. of normal guinea pigs	No. of guinea pigs experimentally infected	Per cent of guinea pigs that developed spontaneous tuberculosis
			per cent
Control................	12	0	0
Cage 1.................	6	6	0
Cage 2.................	12	6	29
Cage 3.................	18	6	60
Cage 4.................	24	6	36
Total...			44

No definite conclusions, therefore, of the effect of crowding on the incidence of spontaneous tuberculosis can be gathered from this experiment.

DISCUSSION.

In the tests as carried out the animals had abundant opportunity for infection. The number of infected animals was kept constant and in all 204 infected animals were observed. At first, an effort was made to replace the tuberculous animals by others that had been infected during an equal time. As the number of deaths increased this, however, was found to be impossible. A careful study of the elimination of tubercle bacilli in the feces has been made in order to obtain some estimate of the degree of exposure

from this source (20). Some animals infected intraperitoneally discharge tubercle bacilli during the 1st week and there is a considerable elimination of tubercle bacilli during the last few weeks of life when a general dissemination of the tuberculosis occurs. A suspension made from one fecal bolus passed by a guinea pig during the period when it eliminates tubercle bacilli is sufficient to produce tuberculosis within 3 months after injection into a healthy pig. Since the average number of such fecal masses passed during 24 hours is 60, a rough estimate of the degree of cage infectivity may be obtained. Nevertheless, in guinea pigs, variation of individual resistance to experimental tuberculosis is considerably greater than is generally supposed. Among very many animals injected in the course of the present study, it has been noted that guinea pigs of the same weight, age and from the same source, inoculated either subcutaneously or intraperitoneally with a fixed quantity of tubercle bacilli taken from the same culture have survived from 6 weeks to 8 months. Those dying after a short period have presented an acute disseminated tuberculosis; those surviving many months, a more chronic fibrotic disease accompanied in two instances with cavitation in the lungs. This individual variation in the span of life and the type of disease has doubtless been accompanied by individual variations in the degree of fecal elimination of tubercle bacilli.

With virulent tubercle bacilli contaminating the food of the normal cage mates, it is readily understood why the intestinal and mesenteric route of infection is so constantly present in instances of spontaneous tuberculosis. In several instances of spontaneous tuberculosis, the lesions were limited to the mesenteric lymph node and to the spleen or to the mesenteric lymph node only. The association in many instances of disease of the cervical as well as of the mesenteric lymph nodes is significant and recalls the bovine tuberculosis of childhood. In a large series of tuberculous guinea pigs injected intraperitoneally, the mesenteric lymph nodes with general dissemination were involved in 8 per cent. In no one instance was the mesenteric lesion particularly prominent. The cervical lymph nodes are even more rarely involved in an experimentally produced disseminated tuberculosis. Although tonsillar tuberculosis and secondary

cervical lymphatic tuberculosis is often found in swine exposed to the feces of tuberculous cattle, I was unable to observe any gross manifestation of involvement of the tonsils in guinea pigs with spontaneous tuberculosis. In studying the route of spontaneously acquired tuberculosis in a small series of guinea pigs exposed to patients suffering with pulmonary tuberculosis, Bartel and Spieler (16) report one instance of a microscopic tuberculous lesion in the pharyngeal wall, but none in the tonsils.

In those instances in which the normal cage mates were exposed to animals with open ulcerative lesions, the type of infection was the same as in instances in which the source of infection was from the feces only. It is probable that if the ulcerations are large enough and the exposure long enough as in the experiments of Römer, 100 per cent of exposed animals will acquire spontaneous tuberculosis.

In only one instance of spontaneous infection among the guinea pigs directly exposed to infected cage mates were the lungs and the tracheobronchial nodes involved with sufficient prominence to indicate that the source of infection was bronchiogenic. The bronchiogenic type of infection is occasionally seen in guinea pigs kept in separate cages but exposed to tuberculous animals in the same animal room. The disease in these instances is comparable to the tuberculosis of the lungs and the hilum lymph nodes in childhood.

The experiments and instances reported in this paper show that spontaneous tuberculosis in guinea pigs is a readily contracted disease. Guinea pigs kept in separate cages in a room with tuberculous animals for a period of more than 3 months should not be used for experimental purposes. If guinea pigs in which suspected material has been injected for diagnostic purposes are killed or die after a period of more than 3 months and tuberculous lesions are found, it is possible that the tuberculous infection was spontaneously contracted. It may be difficult to determine an instance of tuberculosis in a guinea pig as spontaneously acquired, or experimentally initiated if the local lesion at the site of inoculation has healed.

In spontaneous tuberculosis in guinea pigs there is a prominent involvement of either the mesenteric or the cervical lymph nodes or both, secondary to disease of the digestive tract; or of the lungs and

hilum nodes. In my experiments, the portal of entry has been more frequently the intestinal tract. In two instances intestinal ulcers were found in the ileum. These were macroscopic in size. In every instance of spontaneous infection, the intestinal tract in its entire length was carefully examined but it is possible that instances of small ulcerations were overlooked. Further, it is not unlikely that intestinal lesions heal in the course of the infection and are therefore not found at autopsy.

The mesenteric nodes are massively involved and lesions occur in the spleen and liver. The lesions in the spleen are apt to progress more slowly and with considerable fibrosis. Occasionally, the mesenteric lesion may so completely heal that an entire lymph node may become fibrous and lesions which appear in the spleen may be mild and of a chronic type. It is possible that this type of spontaneously acquired disease may go on to complete healing. Not infrequently a mesenteric node was found entirely fibrous and yellowish white, with no other evidence of disease elsewhere in the body. Tuberculin tests during life occasionally showed mild reactions, yet at autopsy, no evidence of disease other than a fibrotic node was found. Such instances were, of course, not included in our series as there was no evidence of the presence of tubercle bacilli either by culture or by animal inoculation. Healed lesions probably occur in guinea pigs exposed to tuberculous infection.

CONCLUSIONS.

1. Normal guinea pigs exposed to tuberculous cage mates infected intraperitoneally and with no cutaneous ulceration readily contract spontaneous tuberculosis.

2. The incidence of spontaneous tuberculosis increases with the intensity and with the duration of exposure.

3. Spontaneous tuberculosis acquired from infected cage mates has with few exceptions the characters of an infection which has entered by way of the digestive tract, disease of mesenteric and cervical lymph nodes being conspicuous.

4. Guinea pigs exposed to tuberculous animals in the same room but not in the same cage may acquire tuberculosis which has the characters of a bronchiogenic infection associated with lesions of the lungs and tracheobronchial lymph nodes.

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A SPECIFIC FLOCCULATION REACTION OCCURRING BETWEEN ALCOHOLIC EXTRACTS OF PNEUMO-COCCI AND ANTIPNEUMOCOCCUS SERUM.*

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INTRODUCTION.

The study of the antigenic properties of lipoidal substances extracted from antigenic material by alcohol, ether, or other fat solvents has included investigation of the significance of lipoidal constituents of the red blood cell in the formation of lysins and agglutinins, the action of alcoholic extracts of organs in the production of heterogenetic antibodies and their reaction with Forssman's antigen, and, finally, of the specific nature of such lipoidal fractions obtained from bacterial cells. Until recently, experimental work on the bacterial cells had been limited almost entirely to the immune reaction in tuberculosis. No attempt is made to review the large amount of work carried out in this particular field by numerous authors, as this has lately been done by Wadsworth, Maltaner, and Maltaner (1). In their study of the complement fixation reaction in tuberculosis, they report a systematic investigation into the distribution of the antigenic properties in tubercle bacillus cultures, which they found to be associated with the acetone-insoluble lipoids.

While the rôle of non-specific lipoids, extracted from various tissues, in the numerous flocculation reactions devised for the serodiagnosis of syphilis, has long been recognized, the action of bacterial lipoids in a specific flocculation reaction had not been made the subject of extensive research until 1924 when Dujarric de la Rivière and Roux

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(2) described flocculation occurring between alcoholic extracts of meningococci and antimeningococcus serum.[1] They found that the combination of antimeningococcus serum with watery emulsions of alcoholic extracts of meningococci, reinforced with tincture of benzoin, would result in macroscopically visible specific flocculation, which they considered to be immunologically different from either the agglutination or the complement fixation reaction. The authors suggest the value of this reaction in the standardization of anti-meningococcus serum. More recently, they (3) have also included the gonococcus in their studies. Georgesco (4) has reported the application of the same method to the serodiagnosis of cerebrospinal meningitis and typhoid fever. Lately, Klopstock (5) has obtained very significant results with alcoholic extracts prepared from pure cultures of *Treponema pallidum* in the complement fixation reaction for syphilis.

While engaged in a study of this flocculation reaction with the meningococcus, during which it was possible to confirm Dujarric de la Rivière's results with serums and extracts prepared in this laboratory,[2] it was decided to investigate whether a flocculation reaction of similar character occurring under essentially the same conditions, could be obtained with alcoholic extracts of pneumococci and anti-pneumococcus serum. Experimental work on this subject was prompted, partly, by the desire to ascertain whether such a flocculation reaction between bacterial lipoids and immune serum was not only species- but also type-specific. It is unnecessary to emphasize particularly in this connection that, in comparison with the pneumococcus, the types of meningococci at present appear to be less sharply differentiated. In the foreground of interest, however, stood the

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[1] An alcoholic extract of meningococci prepared by grinding meningococci with silica and subsequently extracting the bacteria at 37°C. with 96 per cent alcohol was proposed by I. R. Mörch in 1922 (reported by T. Madsen in Reports on Serological Investigations, League of Nations, Health Organization, 1923, 35) as a standard antigen for testing the potency of antimeningococcus serum. This extract, diluted with salt solution, was mixed with decreasing amounts of serum and the mixture incubated for 18 hours at 37°C. A specific precipitation reaction occurred, which was found to provide a basis for the estimation of the therapeutic potency of the serum.

[2] Dr. Dujarric de la Rivière of the Pasteur Institute in Paris kindly provided us with reagents for comparative work with the meningococcus.

study of the relation of this new immune reaction to the therapeutic potency of the immune serum. Obviously, this would meet with obstacles in the case of antimeningococcus serum since no satisfactory test to measure unmistakably the therapeutic potency of this serum has yet been described, and the substantiation of its efficacy by clinical data is extremely difficult and unreliable. This problem would appear to be more accessible to study with the pneumococcus, inasmuch as the standardization of antipneumococcus serum, based on the protection test in mice, reflects with a high degree of accuracy the therapeutic potency of this serum.

EXPERIMENTAL WORK.

Antigens.—The bacterial antigens were prepared by extracting the centrifugalized, washed sediment of 1500 cc. of an 18 hour pneumococcus broth culture in 40 cc. of absolute or 95 per cent ethyl alcohol for a period of 4 weeks, at room temperature. The extracts were then filtered through paper and were ready for use. Antigens obtained by extracting the bacterial sediment for 2 hours with boiling alcohol in a reflux condenser were found to be only slightly weaker in flocculating power than those prepared by the first method. The cultures used were: Three Type I strains—two standard strains, Pneumococcus Type I No. 5 (Neufeld) and Pneumococcus Type I No. 5D (U S. Hygienic Laboratory), and a Pneumococcus Type I strain recently isolated in this laboratory from pneumonic sputum; one Type II strain and one Type III strain, both the latter isolated at and received from the Hospital of The Rockefeller Institute. The extracts used for the major part of the work described in this paper were prepared from Pneumococcus Type I No. 5.

It seemed of interest to obtain some information on the chemical and biological nature of the substances extracted by the alcohol.

5 cc. of extract, Type I, were evaporated, and the dry residue, 6 mg., was resuspended in 2 cc. of physiological salt solution, which produced an almost clear solution. It gave no biuret reaction, but reacted very slightly with Millon's reagent. A Molisch test resulted in a very faint purple color comparable to the one obtained with a 1:500,000 dextrose solution. When the solution was overlaid in 0.3 cc. amounts on 0.3 cc. of each of the tree types of antipneumococcus serum, in no instance did the solution give a precipitin reaction (incubation up to 3 hours at 37°C.). The extracted Type I organisms, on the other hand, suspended in salt solution, were still specifically agglutinated by Type I antipneumococcus serum, although they had become insoluble in bile. This suspension was boiled for a short time, to obtain some of the soluble substances in solution, filtered, and the clear filtrate tested for a precipitin reaction with antipneumococcus serums of

Types I, II, and III. A very distinct type-specific precipitation occurred. In order to obtain information on the antigenic properties *in vivo* of the substances extracted by the alcohol and of the bacterial cells after extraction, 20 cc. of alcoholic extract were evaporated, and the dry residue was resuspended in 15 cc. of salt solution. Simultaneously, the bacterial cells after extraction, representing the treated sediment of 750 cc. of broth culture, after several washings, were suspended in 15 cc. of salt solution. One rabbit was given the total volume of the first preparation divided into four subcutaneous injections; another rabbit, the total volume of the second preparation similarly divided. Both animals were bled on the 5th day following the last injection. While the serum from the rabbit which had received the emulsion of the residue of the alcoholic extract gave neither an agglutination nor a precipitin reaction, the serum of the rabbit treated with the bacterial cells after extraction with alcohol, agglutinated faintly up to a 1:2 dilution and gave, undiluted, a distinct precipitin ring with the filtrate of a 48 hour autolyzed pneumococcus broth culture. The two rabbits were then tested for the degree of immunity developed, by the intravenous injection of 0.001 cc. of a highly virulent pneumococcus culture which had been passed through rabbits for a number of years. This dose, which killed a normal control rabbit in 48 hours, killed the rabbit which had received the emulsion of the residue of the alcoholic extract in 72 hours, while the other animal, immunized with the alcohol-treated bacteria, survived. The same animal, when injected 4 days later with 0.1 cc. of the same culture, was fully protected.

Serums.—The serums used for the tests were monovalent antipneumococcus serums obtained from horses immunized by various methods. Incidentally, a small number of immune serums from rabbits immunized with the whole pneumococcus culture for various periods of time, were tested. Finally, it has been possible to examine several samples of serum from pneumonia convalescents, obtained through the courtesy of Dr. Cole from the Hospital of The Rockefeller Institute in New York City and from Dr. Ordway of the Albany Medical College.

The Test.—In setting up the test, Dujarric de la Rivière's original method was modified in many respects. The addition of an unspecific reinforcing agent to the specific alcoholic bacterial extracts proved to be indispensable in order to make the flocculation reaction macroscopically readily visible. A 10 per cent alcoholic extract of Sumatra benzoin[3] was used for this purpose after it had been found that lecithin and cholesterol, although capable of reinforcing the intensity of the reaction considerably, did not induce as rapid a flocculation as did the benzoin.

⸳ [3] It would seem advisable to use no benzoin tincture which is older than 2 months, since evidence of deterioration was noted after that time. The bacterial antigens, on the other hand, appear to be stable for a longer period.

The technique of the test as finally adopted is briefly as follows:

To one part of benzoin tincture are added twelve parts of alcoholic bacterial antigen. This initial mixture is suspended in 0.85 per cent salt solution by adding quickly 25 cc. of the salt solution to 0.4 cc. of the initial mixture. It is important to add the diluent very quickly. Experiments in which buffered salt solutions of different pH values were used in preparing this suspension, indicated a neutral medium as the most desirable for the reaction. The colloidal suspension thus obtained is slightly opaque and just on the verge of spontaneous flocculation; if left standing at room temperature or shaken vigorously, it will flocculate by itself. In order to insure conditions of maximum sensitivity, it is necessary to determine for each new antigen and new benzoin tincture the optimum balance between bacterial antigen, benzoin, and salt solution. A constant volume of 2.5 cc. of this suspension is mixed with varying amounts of serum, starting with 0.5 cc. and ending with 0.05 cc. It is not advisable to test smaller amounts than 0.05 cc. because it was found that less than 0.05 cc. of normal serum is insufficient to stabilize the suspension; the comparable range of specific flocculation is thus limited to the amounts indicated. The tubes are incubated, half immersed in a water bath at 40°C. since this temperature was found to provide conditions of the greatest sensitivity, maintaining at the same time the highest degree of specificity; it was observed that normal horse serum flocculated slightly above 46°C. Readings are made after $\frac{1}{2}$ hour, 1 hour, and 2 hour intervals to determine the degree of flocculation. Occasionally, another reading is made on the following day.

Results.—It was found that a number of different antipneumococcus, Type I, Type II, and Type III serums, when tested in the manner described, gave a flocculation reaction of varying intensity with their homologous alcoholic bacterial antigens. While a great many of the fresh Type I serums gave a marked flocculation with Type I antigens; two older Type II serums and one older Type III serum, the only serums of these types available, reacted only faintly with their respective antigens. The species specificity of this reaction was controlled (1) by testing several normal horse serums as well as other immune serums, such as, antimeningococcus serum and antidysentery serum, with the pneumococcus antigen, (2) by testing alcoholic extracts of other microorganisms prepared, in a similar way, with antipneumococcus serum. In no case was flocculation observed under such conditions within the limits of the experiment.

In order to determine whether this reaction exhibited also type specificity, pneumococcus antigens, Types I, II, and III, were tested with the heterologous type serums. Some slight cross-reactions oc-

curred between Type II and Type III antigens and Type I serum as recorded in Table I, but no overlapping was noted with Type I antigen.

It was soon found that a number of Type I serums older than approximately 1 year, flocculated slightly, if at all, in spite of the fact that they still agglutinated and protected very well. It must remain undecided at present whether serums lose their flocculating power by aging, because no serum giving flocculation has yet been observed for longer than about 4 months. For the same reason, it is impossible to state whether the weak reactions obtained with two Type II serums and one Type III serum with their homologous antigens are attributable to the age of the serums, which were between 1 and $2\frac{1}{2}$ years old, or whether flocculation runs parallel to the protective action of antipneumococcus serum in general, having no relation to the agglutinative titer. As is usually the case, these Type II and Type III serums had very little, if any, protective action, while their agglutination titer was approximately the same as that of the Type I serums.

The results of these tests are tabulated in Table I.

A number of serums from rabbits immunized with pneumococcus, Type I, were tested for flocculation. While the reactions with these rabbit serums, which had a comparatively high agglutination titer, were very weak, the tests were too few to be of much significance. There is some reason to believe that, after prolonged immunization, the serum may acquire definite flocculating power.

The results obtained with the pneumonia convalescent serums are still too scanty to warrant a detailed report, although definite flocculation occurred in several instances with serum taken as early as the 2nd day of the disease. Occasionally, however, a very slight flocculation with the largest amount of normal human serum tested, 0.5 cc., was noted. In this connection, it should be recalled that Wadsworth (6), in his early work, obtained a precipitin reaction with high dilutions of convalescent pneumonia serums by using extracts of pneumococci. The centrifugalized fresh pneumococcus cells had first been shaken with hypertonic salt solution to increase the plasmoptysis, the extract then being made isotonic by the addition of water. He also observed a reaction with serums from apparently

healthy persons, in a dilution of 1:10, after 16 hours. It is hoped to improve the technique in future work so that significant results may be secured.

Further study was directed to a closer investigation of the nature of this flocculation reaction. It was found that supernatant serum which had flocculated, would not flocculate again, while the agglutination titer and the protective action of such a serum had remained practically unaffected; the solution of the flakes in salt solution up to the original volume released no protective action. Next, the effect of absorption was studied. It appeared that Pneumococcus Type I serum which, after absorption for 6 hours at room temperature with Type I organisms, had lost completely its agglutinative action, showed no flocculation whatsoever for the first 2 hours of incubation. Left overnight at room temperature, there was, however, considerable flocculation, although much less than occurred in the non-absorbed serum even after 2 hours' incubation. Later, the effect of heating for $\frac{1}{2}$ hour and 1 hour at 56°C. was studied in two different Type I serums. The results obtained did not agree. While heating for $\frac{1}{2}$ hour impaired the flocculating power of one serum considerably and heating for 1 hour completely destroyed it, the other serum, after being heated for $\frac{1}{2}$ hour, flocculated almost as well as the non-heated serum and even heating for 1 hour did not destroy its flocculating power. The agglutinative action of these two serums was similarly affected by heating as was the flocculating power. The addition of complement, in the form of fresh normal horse or guinea pig serum, did not reactivate the heated serum nor did the addition of complement to old serums improve their flocculating power.

Finally, the relation of this flocculation test to the therapeutic potency of Type I antipneumococcus serum was studied. Over forty serums obtained from ten different horses at various stages during immunization were tested for flocculation, and the results of these tests were compared with the protective titer ascertained by the routine method of standardization in mice. Although it had already been observed that the serums in repeated tests exhibited approximately the same flocculating power on different days, a serum which flocculated well was chosen as a standard and run along simultaneously with normal horse serum with each test, as a control. The results are tabulated in Table II.

TABLE I.

*Experiments on Type and Species Specificity of Flocculation Reactions with Alcoholic Extracts of Pneumococci.**

Pneumococcus Type I antigen

Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Pnc. Type I Serum 312	1.7.26	2+	3+	4+	2+	2+	3+	±	1+	1+
" I " 313	1.26.26	2+	3+	4+	2+	3+	4+	2+	3+	3+
" II " 93	10.22.23	–	–	–	–	–	–	–	–	–
" II " 85	7.16.23	–	–	–	–	–	–	–	–	–
" III " 62	1.4.25	–	–	–	–	–	–	–	–	–
Normal Horse Serum (H. 144)	4.24.25	–	–	–	–	–	–	–	–	–
" " " (H. 140)	2.5.24	–	–	–	–	–	–	–	–	–
" " " (H. 140)	2.2.26	–	–	–	–	–	–	–	–	–
Antimeningococcus Serum 145	3.3.25	–	–	–	–	–	–	–	–	–
Antidysentery Serum 75	2.7.25	–	–	–	–	–	–	–	–	–

Pneumococcus Type II antigen

Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Type I Serum 312	1.7.26	–	±	1+	–	±	±	–	–	–
" I " 313	1.26.26	–	±	1+	–	±	±	–	–	–
" II " 93	10.22.23	–	±	1+	–	±	–	–	–	–
" II " 85	7.16.23	–	±	±	–	–	–	–	–	–
" III " 62	1.4.25	–	–	–	–	–	–	–	–	–
Normal Horse Serum (H.144)	4.24.25	–	–	–	–	–	–	–	–	–

| | | Pneumococcus Type III antigen | | | | | | | | | Antigens from other microorganisms tested with Type I Antipneumococcus Serum 312 | | | | | | | | | |
| | | Mar. 9 0.5 cc. of serum incubated for | | | Mar. 9 0.3 cc. of serum incubated for | | | Mar. 9 0.1 cc. of serum incubated for | | | | Mar. 10 0.5 cc. of serum incubated for | | | Mar. 10 0.3 cc. of serum incubated for | | | Mar. 10 0.1 cc. of serum incubated for | | |
Serums	Date bled	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	Antigens	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Type I Serum 312	1.7.26	−	−	±	−	−	−	−	−	−	*Streptococcus viridans*	−	−	−	−	−	−	−	−	−
" I " 313	1.26.26	−	−	1+	−	−	−	−	−	−										
											Micrococcus catarrhalis	−	−	−	−	−	−	−	−	−
" II " 93	10.22.23	−	−	−	−	−	−	−	−	−										
" II " 85	7.16.23	−	−	−	−	−	−	−	−	−	Meningococcus 10-B	−	−	−	−	−	−	−	−	−
											" W 30-B	−	−	−	−	−	−	−	−	−
" III " 62	1.4.25	±	1+	1+	±	1+	1+	−	−	−	" 46-B	−	−	−	−	−	−	−	−	−
											" 79-B	−	−	−	−	−	−	−	−	−
Normal Horse Serum (H. 144)	4.24.25	−	−	−	−	−	−	−	−	−										
											Alcohol	−	−	−	−	−	−	−	−	−

*The intensity of flocculation is designated as follows:

− = diffuse opacity.

Tr. = trace (faintest granulation).

± = slight granulation.

1+ = small flakes.

2+ = medium sized flakes.

3+ = large flakes.

4+ = heavy flocculent precipitate at the bottom leaving supernatant fluid clear.

TABLE II.

Comparison between Flocculation Reaction, Agglutination Titer, and Protective Action of Different Type I Antipneumococcus Serums.

Horse and bleeding Nos.	Serum No.	Immunization	Date bled	Dates of flocculation tests 1926		Flocculation after 1 hr.'s incubation at 40°C.			Date of agglutination test	Agglutination after 2 hrs.' incubation at 37°C.				Date of protection test	Protective action	
						0.5 cc.	0.3 cc.	0.1 cc.		1:10	1:20	1:40	1:80		Standard serum	Test serum
															Culture 0.2 cc. protected	
															cc.	cc.
143-10	271	11 mos.	7.24.24	2.5	2.9	Tr.	Tr.	–	9.11.24	3+	3+	–	–	9.17.24	0.1	0.2
–11	273	11 "	7.29.24	2.5	2.9	2+	2+	±						9.17.24	0.1	0.2
–12	279	1 yr. 3 mos.	1.3.25	2.5 2.9	2.11	Tr.	Tr.	Tr.						1.7.25	0.1	0.3
152-13	270	1 yr.	7.16.24	2.5	2.9	–	–	–	9.11.24	2+				7.31.24	0.1	0.1
–14	272	1 "	7.29.24	2.5 2.9	2.11	±	Tr.	Tr.						9.17.24	0.1	0.2
190-3	289	4 mos.	4.22.25	1.29	2.5	±	–	–	2.1.26	4+	2+			5.13.25	0.1	0.1*
180-4	290	5 "	5.6.25	1.29	2.5	1+	Tr.	Tr.	7.17.25	3+	3+	1+	1+	5.28.25	0.2	0.1
193-3	303	105 days	11.7.25	2.21	2.24	±	–	–	3.18.26	3+	2+	1+	±	11.11.25	0.1	0.1*
–4	309	136 "	12.8.25	2.21	2.24	3+	2+	1+	2.1.26	3+	3+	2+	–	12.9.25	0.1	0.2
–5	310	142 "	12.14.25	2.21	2.24	3+	2+	2+	3.18.26	3+	3+	3+	2+	12.16.25	0.1	0.1
–6	311	164 "	1.5.26	2.21	2.24	3+	3+	2+	3.18.26	3+	3+	3+	1+	1.6.26	0.1	0.1
–7	313	185 "	1.26.26	2.21	2.24	3+	3+	3+	3.18.26	3+	3+	3+	–	1.27.26	0.1	0.1
–8	316	207 "	2.17.26	2.21	2.24	2+	3+	1+	3.18.26	3+	2+	2+	–	3.4.26	0.1	0.1
–9	318	221 "	3.2.26	3.5	3.7	2+	1+	1+	3.18.26	3+	3+	2+	–	3.16.26	0.2	0.2
161-3	302	105 "	11.7.25	2.11	2.24	2+	1+	–	3.18.26	3+	2+	±	–	11.11.25	0.1	0.1*
–4	307	135 "	12.7.25	2.11	2.24	1+	Tr.	–	3.18.26	3+	3+	1+	–	12.9.25	0.1	0.1**

−5	312	166 days														
−6	314	188 "	1.7.26	2.11	2.24	3+	2+	1+	3.18.26	3+	2+	2+	−	1.13.26	0.1	0.1
−7	317	215 "	1.29.26	2.11	2.24	3+	3+	1+	3.18.26	3+	3+	1+	−	2.10.26	0.1	0.1
−8	322	231 "	2.20.26	3.5	3.17	1+	±	±	3.18.26	3+	3+		−	3.4.26	0.1	0.2
−9	323	244 "	3.13.26	3.17	3.19	±	±	−	3.18.26	3+	3+		−	3.16.26	0.2	0.1
−10	327	263 "	3.27.26	4.9		−	−	−	4.28.26	3+	2+		−	3.31.26	0.1	0.1*
			4.16.26	4.20		±	±	−	4.28.26	4+			−	4.21.26	0.1	0.1
201−1	306	111 "	11.13.25	2.5	2.9	1+	±	−	3.18.26	3+	1+	±	−	11.18.25	0.2	0.1
−2	308	136 "	12.8.25	2.5	2.9	2+	1+		2.1.26	3+		−		12.9.25	0.1	0.1
207	Norm. bl.	0														
	1st trial bl.	38 "	12.19.25	2.23	2.26	−							−			
	2nd " "	56 "	1.26.26	2.23	2.26	−							−			
−1	320	82 "	2.13.26	2.23	2.26	−		−	3.18.26	3+	1+	−		2.3.26	0.3	0.1**
−2	325	103 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	3+		−	2.17.26	0.1	0.1**
−3	328	124 "	4.1.26	4.9				−	4.28.26	3+	3+	±	−	3.16.26	0.2	0.1
−4	331	147 "	4.22.26	4.22		1+	1+	±	4.28.26	4+	3+	1+	−	4.14.26	0.1	0.2
			5.13.26	6.1		1+	1+	1+	5.19.26	3+	3+		−	4.26.26	0.1	0.2
150	Norm. bl.	0												5.19.26	0.2	0.2
	3rd trial bl.	64 "	11.27.25	2.23	2.26	−							−			
	4th " "	73 "	1.30.26	2.23	2.26	−							−			
−1	315	82 "	2.8.26	2.23	2.26	−			4.28.26	3+	1+	−		2.3.26	0.2	0.1
−2	319	96 "	2.17.26	2.23	3.17	Tr.		−	3.18.26	3+	3+	−		2.10.26	0.2	0.1
−3	324	124 "	3.3.26	3.15				−	3.18.26	3+	2+	1+	−	2.24.26	0.1	0.1
			3.31.26	4.9		±	2+	1+	4.28.26	3+	3+	±	−	3.10.26	0.1	0.1
151	Norm. bl.	0				2+								11.15.25	0.1	0.1†
	1st trial bl.	38 "	12.19.26	3.5	3.15	−							−			
	2nd " "	56 "	1.26.26	3.5	3.15	−										
−1	321	82 "	2.13.26	3.5	3.15	−			3.18.26	3+		−		1.27.26	0.1	0.1**
−2	326	103 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	2+	1+	−	2.17.26	0.1	0.1**
−3	329	124 "	4.1.26	4.9		−	−	−	4.28.26	4+	1+		−	3.16.26	0.2	0.2
−4	330	147 "	4.22.26	4.29					4.28.26	4+	2+		−	4.21.26	0.1	0.1
			5.13.26	6.1		±	±	±	5.19.26	4+	2+	±	−	4.26.26	0.1	0.1
														5.19.26	0.2	0.2

* Protected partially. ** Did not protect.

† Earlier tests of the serum, while irregular, suggested its value to be approximately equal to that of the standard serum.

In studying Table II, Horses 193 and 161 attract particular attention, inasmuch as it had been possible to follow a number of bleedings taken between the 3rd and the 8th or 9th month of immunization, and to compare the results of the protection test in mice and the agglutination titer with the flocculating power as measured by the test described. A relationship between the flocculating power of the serum from these horses and the protective action is suggested. In the case of Horse 161, a marked decrease in the flocculating power of the serum occurred before the end of immunization. This horse was in very poor physical condition when bled out. It is also of special interest to note that Horse 193 developed endocarditis toward the end of immunization, which condition apparently did not affect the protection titer of the serum, but there was a slight drop in the flocculating power. Horses undergoing active immunization by the intravenous inoculation of living virulent pneumococci frequently develop lesions in the heart valves, as first described by Wadsworth (7), in 1919, and yet their serums possess high protective action in mice against virulent pneumococci. In Horse 150, the flocculating power developed very much more slowly than the protective action and, in Horses 151 and 207, even at the end of the 5th month of immunization, there was only slight flocculation, although three previous bleedings of Horse 151 and two previous bleedings of Horse 207 had given a protection value at least equal to that of the standard serum. The agglutinative activity, as evidenced especially by Horses · 207, 150, 151, appears much earlier than either the protective antibodies or the flocculating power, after which they run a somewhat parallel course (Horses 193 and 161). It is apparent that, in spite of frequent general agreement between flocculating power and protective action, there remain considerable discrepancies among individual serums which will have to be elucidated by further studies.

DISCUSSION.

Although, at present, it is impossible to give a lucid explanation of the mechanism of the reaction described, it would seem that an immune reaction, exhibiting strict species and a high degree of type specificity may be obtained with substances extracted from the pneumococcus cell by absolute or 95 per cent ethyl alcohol. The method

of preparing the alcoholic antigen does not preclude the possibility that, besides lipoidal substances, certain impurities of protein or carbohydrate character may have been carried over into the extract. The extracted material so far has been obtained only in such small amounts that an exact chemical analysis has not been possible; also, no other solvents have been used. It would seem, however, from the tests done that only traces of substances of protein or carbohydrate nature can have been present in the extract. The reaction apparently depends essentially upon the presence of bacterial lipoids.

In this connection, there comes to mind the work of Avery and Heidelberger (8–10), who, basing their conclusions upon precipitin tests, attribute the type specificity of the pneumococcus to a substance of carbohydrate nature while a nucleoprotein is designated as the carrier of the species specificity of the organism. On the other hand, Zinsser and his associates (11–14) have prepared, from various bacteria, including also the pneumococcus, substances—residue antigens—which are free from coagulable protein and are capable of reacting specifically in a precipitin test with the homologous antiserum. Both of these substances, the polysaccharide of Avery and Heidelberger and the residue antigen of Zinsser, were found by the respective authors to be incapable of producing antibodies. Perlzweig and Steffen (15), in studying different fractions of the pneumococcus for their immunizing effect in mice, found the alcohol-soluble fraction of a trypsin digest of Pneumococcus Type I organisms as active as the ordinary vaccine of heat-killed pneumococci in affording protection, while they failed to obtain immunity with alcohol, ether, chloroform, and acetone extracts made from intact, dried pneumococci. They also state that among the various antigenic solutions which were tested for a precipitation with immune serums, some of the potent alcohol-soluble immunizing antigens failed to react, while, in other instances, precipitation was observed. In this connection, the experiments of Brotzu (16), who found ether-extracted pneumococci to lack antigenic activity in the immunization of rabbits, are of interest.

It remains for further study to coordinate the results obtained with the alcohol-soluble substances described in this paper with those described by the investigators cited above.

Although the type specificity of the reactions is quite marked in some of the tests, more particularly with Type I antigen; in others, it is not quite so definite. Similarly, although the results of the flocculation reactions in general appeared to correspond to those of the protection test, in many instances they were divergent. Since the protection test varies so markedly with the virulence of the

pneumococcus, it is not possible to determine as yet which test yields the more uniform quantitative results. The extent to which differences in the activity of the several antigens and serums figure in the test and whether or not the test can be more accurately standardized for quantitative determination, will require special investigation. Nevertheless, the results obtained so far indicate the desirability of further study of this reaction in connection with the type diagnosis of pneumonia and suggest its value in the standardization of Type I antipneumococcus serum.

SUMMARY AND CONCLUSIONS.

1. A flocculation reaction has been described which occurs between alcoholic extracts of pneumococci and antipneumococcus serum.

2. The reaction appears to be species-specific. It is not strictly type-specific, as slight or moderate cross-reactions occurred between Type I serums and Type II and Type III extracts.

3. The flocculating power of the serum from five horses undergoing immunization with pneumococcus, Type I, did not develop to any extent before the end of the 4th or 5th month.

4. In the case of two of these horses in which it was possible to carry out parallel tests on a larger number of subsequent bleedings until the end of immunization, some relationship was suggested between the flocculating power and the protective titer as ascertained by the routine method of standardization in mice.

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AN EXPERIMENTAL STUDY OF GYE'S CANCER THEORY.

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The experiments of Gye (1) on the etiology of malignant growth, based on a study of the Rous chicken sarcoma, have attracted much attention. His conclusions offer an explanation not only of the cancer problem, but also, indirectly, of that of the nature of other types of filterable viruses. A repetition of his work has been undertaken in a number of different laboratories. At least four reports of such work have already reached the literature,—those of Murphy (2), Harkins, Schamberg, Kolmer, and Kast (3), Flu (4), and Cori (5). The results published in these papers differ widely among themselves, and all are at variance with those of Gye.

Gye believes that tumors in general are caused by a filterable virus probably common to all tumors irrespective of variety or source and capable of growth *in vitro.* Under experimental conditions, this virus is unable to infect without the aid of another factor which ruptures the defense of the cell. In the case of filterable chicken tumors, such an agent is present, which is readily separable from the cells. He calls this the "specific factor," since it is effective only in chickens to produce always the same type of tumor from which it is derived.

These conclusions are based, primarily, on two types of evidence. In the first place, Rous sarcoma filtrates, when centrifuged for an hour at 9000 revolutions per minute, are separated into a sediment and a supernatant fluid, neither of which, alone, appears to be capable of producing a tumor in chickens, but, when injected together, will do so. Gye admits that it is difficult to get clear-cut results with this technique, and Flu, who is the only one to have published his experiences in attempting to repeat this phase of the work, obtained one positive result out of three experiments, but feels that it is unwarranted to draw definite conclusions from this without further controls. The second and main line of

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proof is more difficult to carry out, and subject to more theoretical and practical criticism. Specific factor from the Rous sarcoma is obtained by treating the filtrate with chloroform under definite conditions, which is supposed to kill the virus. Preparations of the latter are supplied by cultures of the Rous sarcoma or of various rat and mouse tumors, and, in one case, of a human mammary carcinoma. In all of Gye's published protocols the injection of either factor alone, in a quantity of 1.0 cc. into a chicken, gave no result, while a mixture of 0.5 cc. of each always produced a large tumor in 2 weeks or less.

Gye's primary tumor cultures were made by adding 1 gm. of the tumor to 5 cc. of Hartley's broth plus 0.2 per cent KCl, to which was added 1.0 cc. of rabbit serum. Subcultures were made by transferring a loopful of a primary culture, after 4 days' anaerobic cultivation at 36° in a McIntosh and Fildes jar, to similar media containing a fragment of 10 to 14 day chick embryo. Subcultures of the Rous sarcoma as far as the twelfth generation were found to be active, while only primary cultures of the mammalian tumors were used.

Gye does not include protocols in his paper in which uninoculated tubes of Hartley's broth containing rabbit serum and embryonic tissue are used as controls after incubation. Murphy (2) reports on three short experiments in which successful reactivation of the chloroformed filtrates by means of tumor cultures was shown and in which reactivation with such uninoculated controls was also positive, and believes the results are due to an acceleration of a partially destroyed agent by means of some substance common to rapidly growing tissues such as tumor, embryo, or placenta.

Cori (5) reports one similar experiment out of eleven, the other ten being negative. Flu (4) carries Murphy's criticism a step further and reports successful reactivation not only with tumor cultures and embryo "cultures," but also with similarly prepared cultures of liver and kidney tissues from both chickens and guinea pigs.

On the other hand, Harkins, Schamberg, Kolmer, and Kast (3) obtain different results. If enough chloroform is added to the Rous sarcoma filtrates so that tumors are not produced by the injection into chickens of this substance alone, the addition of tumor cultures made according to Gye's methods does not restore infectiousness.

It should be stated at this time that the results to be described in this paper are in accord with those of Harkins, Schamberg, Kolmer, and Kast. Before entering into a consideration of the possible reasons for the divergence of results in different laboratories our experiments may be presented.

Methods.

The writer wishes to acknowledge with thanks the assistance obtained in several letters from Dr. Gye, and in conferences with Dr. Max Cutler. It has been our aim to follow Gye's directions in attempting to repeat his experiments. These are commented upon only where necessary.

The Tumor.—Dried tumor of the Rockefeller chicken tumor No. 1 (Rous sarcoma), marked "Desiccation 31, VII, 1, 25" was kindly supplied us by Dr. Rous. Injection of a small quantity of this, suspended in Ringer solution, into the breast muscle of chickens led to the production of tumors in about 14 days, with death in 3 to 4 weeks. From such tumors, inoculations of cells, desiccated or glycerolated tissue or filtrates have uniformly produced tumors in 1 to 2 weeks. Metastases have been frequent, but not invariable, to liver, heart muscle, and lungs. In many cases they have not been looked for, since the local tumor has been the important point in the work. Microscopic examination has also not been made of all tumors, but when done, the picture agreed with the earlier published descriptions of the tumor. We have had little difficulty with contaminated cultures. Instead of soaking the chicken in lysol, before excising tumor tissue for culture, we have found it more convenient, and equally satisfactory, to kill the chicken quickly, dip for a moment in boiling water, strip off the feathers for some distance around the site of the tumor, and paint the skin with strong iodine.

Gye and Andrewes (6) have published the interesting observation that the Rous sarcoma occasionally undergoes a change in properties becoming "non-filterable." The tumors we have used have not invariably been tested, but they have at intervals passed through medium Berkefeld filters (no more failures than one would expect in this type of experiment), and have survived glycerolation for 3 days or longer.

The Chickens.—Most of the experiments have been carried out on pure bred Barred Rock chicks of 6 to 8 weeks of age. While the tumor usually appears in 10 days, there have been instances of much later development, and to guard against overlooking tumors, the chickens have been kept for 3 months after inoculation, or until death from intercurrent disease occurred.

Sand Filtrates.—Made exactly as described by Gye and Andrewes (6) and filtered through sand and paper pulp in an apparatus similar to his. It has usually been necessary to use gentle suction in order to get the material through in a reasonable length of time, and the Ringer suspension has occasionally been centrifuged for a short time to hasten subsequent filtration. The sand filtrates have, with a single exception, proved to be highly infectious, producing rapidly growing tumors in quantities of from 0.1 cc. to 0.01 cc. or even less.

While the proportionality which Gye stresses between quantity injected and size and rate of growth of tumor holds to a certain extent, it has not impressed us as being sufficiently striking to serve as the basis for a theory that a chemical substance and not a living agent is involved. In point of fact, while Gye bases the reasoning for his experiments upon this observation, he nevertheless always uses a quantity of virus culture equal in amount to the chemical specific factor, whereas, if his hypothesis holds, a trace should be sufficient.

After a number of preliminary experiments in which ordinary chloroform was used, Merck's reagent chloroform was redistilled, the middle third saved and kept

in a dark bottle in the ice box, in order to avoid the action of possible impurities on the filtrate. It was found that tumor filtrates varied considerably in their resistance to the action of the reagent. A number of experiments were wasted in attempting with a single quantity of chloroform to produce a suitable specific factor. The results showed almost uniformly either a development of tumor with both specific factor control and mixture, or no tumor with either. Gye states that he uses "a few drops" of chloroform, sufficient to saturate, but not a large excess. We found that filtrates could be saturated, as shown by the presence of droplets of the reagent at the bottom of the tube after mixing, and still be infectious after 3 hours' incubation at 37°C. A larger excess led to complete inactivation so that it was not possible to reactivate by any virus tried and, as stated above, the amounts necessary to bring about these results varied from one tumor to the next.

In order to avoid this variation, desiccated tumor material was used for the preparation of some of the chloroform filtrates, since it must contain both "specific factor" and "virus." By preparing a quantity of this from a single large tumor, it should be possible to have uniform material for a number of experiments. Three or four different portions of the same sand filtrate were used in each experiment, and varying amounts of chloroform measured in with a fine, graduated pipette instead of being added in drops. It was noted that where Gye's directions for adding the chloroform were followed, i.e., allowing it to run down the side of the tube so that a portion floated on the surface, followed by incubation at 37° for half an hour before mixing, most of that which floated was lost by evaporation, and the quantity subsequently mixed with the filtrate was less, and in the case of quantities as small as 0.05 cc., markedly less than it was intended to add. In some of the experiments we therefore mixed the chloroform with the fluid immediately after adding by means of drawing up repeatedly in a capillary pipette, and again mixed at the end of 30 minutes in the same way.

At the end of 3 hours the chloroform was removed by suction, by means of an oil pump. A great deal of frothing always took place, which made it necessary to transfer the fluid to a suction flask before carrying out this step, and to apply the suction carefully for some minutes. After a time the frothing ceased, and the full force of the pump could be used to obtain violent boiling. In transferring the material to the suction flask, the greater part of the excess chloroform usually remained adhering to the test-tube as an emulsion.

The protocols of the experiments in which desiccated tumor tissue was used will be given later, together with a discussion of the results. Since in general the experiments were not satisfactory, further work was done, following the same plan by again using filtrates from fresh tumors.

Virus Preparations.—Several different lots of Hartley broth were used in the course of the work. Beef muscle, ox heart, and horse muscle were all tried, but no difference was apparent. Gye, in a personal communication, asserts that not all rabbit sera are equally good, and that it is impossible to tell, except by trial,

which ones are suitable. He says, further, that in carrying a strain of the virus along in culture, at some stage the organism may die out, producing what he calls a "stop." Further subcultures from such a tube always produce negative results. Primary cultures of either chicken or mammalian tumors, he says, do not require rabbit serum, but subcultures will never succeed without it. These facts make it extremely difficult to know whether negative results may not be due to faulty cultures, and there seems to be absolutely no way to control them. In the early stages of the work "passage strains" of the Rous sarcoma were used. It was readily shown that primary cultures were infectious, and aerobically grown primary cultures which Gye occasionally used as virus (oxidation, according to his theory, destroying the specific factor rather quickly) usually gave positive controls in our hands. Upon receiving the above details as to cultures from Dr. Gye, we changed the character of our cultures, omitted the rabbit sera, and·used only primary cultures of the Jensen rat sarcoma, or some other transplantable rat or mouse tumor. Controls containing chick embryonic tissue or rat or mouse embryo and placenta have been frequently used.

After 48 hours' anaerobic incubation, the jar is opened and the tubes examined grossly and by streaking out on blood agar, for contaminations. They are then returned to the jar and incubated 2 days longer before use. At the time of using, any contaminated tubes, as shown by the blood agar cultures, are discarded, the remainder are examined by stained smear. Several tubes of each type of culture are always made and before use the uncontaminated tubes of the same material are mixed together. This would seem to be the surest way of getting a serviceable virus preparation. In some cases the jar has not been opened at the end of 2 days, but inspection and stained smear at the time of use have been relied upon. Cultures made on blood agar plates at the time of using, as a check on the smear, usually, but not invariably remain sterile. There is, in general, little difficulty about contamination when the ordinary precautions of bacteriological technique are followed. If, however, a tumor is used which has begun to ulcerate, practically every tube is unavoidably contaminated.

Injections and Method of Control.—A small amount of sterile Kieselguhr is added to each preparation and the tube shaken before drawing up the material into the syringe. Where mixtures of specific factor and virus are made, the solutions are pipetted separately into a clean tube, the greatest care being taken not to contaminate one tube with the contents of another. Injections have been made into the muscles of the breast, legs, and upper wing.

The question of a suitable method of control is a most puzzling one, and at the same time, is of the utmost importance. There is a certain amount of difference in susceptibility to small quantities of the agent even in young Barred Rock chicks. Hence, one can never be sure that a negative control with 1.0 cc. in one chick, and a take with 0.5 cc. in another chick is significant unless it happens consistently. Beside the individual resistance, there is some other factor which the writer is unable to define which obscures the matter. For example, the same

quantity of material of reduced infectivity injected into both sides of the breast of a number of chickens may give in one chicken no takes, in another takes on both sides, and, what is still more important, in still others *takes on one side and not on the other*. Probably some such element as the quantity of Kieselguhr, the degree of trauma, the relation of the injected material to large vessels or lymphatics, or the amount of hemorrhage, all factors beyond control, is the determining point. This statement will be illustrated in later protocols. Now, if controls are done in separate chickens, considerable numbers must be done or they are without significance. If they are done in the same chickens, the work is open to theoretical criticism for this reason: Granting that two substances, A and B, are necessary to produce the lesion, if A is injected at one site, B at another, and A and B at a third, it is conceivable that A or B may not remain localized, but may enter the circulation, be carried to another site, and finding there B or A substance, produce a false positive control. One has the choice of two evils. Probably the only safe procedure is to use both types of controls, and do the experiment on a large scale, so that numbers will offset individual and chance variations.

Criteria for Judging Results.—From what has been said, it is evident that one cannot expect every experiment of this type to be satisfactory. It is equally evident, if the theory is to hold, that in a single experiment the results should be consistent. That is to say, if thirty or forty chickens are done, many of them duplicates or triplicates, it should be evident in viewing the protocols that the same thing is taking place throughout that particular experiment. It is entirely without significance to pick out two or three chickens and, neglecting the others, accept a probably fortuitous result as an experimental fact. *It is true that there may be a perfectly reasonable difference of opinion as to the significance of such an experiment, but one should have the opportunity of drawing his own conclusions from a complete protocol.* We do not mean, necessarily, that every chicken used should be accounted for, although by doing this very thing, Harkins and his collaborators have presented a valuable piece of work, but within a given experiment, at least, all should be described, and where only a few chickens are used enough similar experiments should be done and described to show whether the result is an accident or not.

This point of view has been reached after using a large number of chickens in experiments of this type. Many of these, in the earlier part of the work, where only a few were used at a time, need not be presented here, since they were frankly negative in results, either from the use of too much or too little chloroform. Commencing with the work with desiccated tumor in which more chickens were used, each experiment will be summarized. Unfortunately, an epidemic of coccidiosis killed a number of the chicks in the experiment of May 28 and it has been impossible to completely eliminate the infection from the flock. Evidence of it has appeared in all subsequent experiments as occasional deaths with extreme emaciation.

Experiment April 14, 1926.—Desiccated tumor was used as a source of specific

factor. A large rapidly growing tumor was excised, from which 60 gm. of fairly healthy tissue was obtained. This was minced and frozen. It was then transferred to a desiccator containing P_2O_5, the air was exhausted with an oil pump, and the desiccator then placed in an ice box until the material was completely dry. It was then ground, and preserved over P_2O_5 in the dark. 0.75 gm. of this dried material, considered to be equivalent to about 5.0 gm. fresh tumor, was ground with sand and 100 cc. Ringer solution, filtered through a sand filter, and chloroformed in the usual manner. Three quantities of 20 cc. each were prepared, 0.1 cc., 0.6 cc., and 1.0 cc. respectively of chloroform being used, and the preparations designated as Cl_1, Cl_2, and Cl_3. In this preparation Gye's directions for chloroforming were followed. The virus used was a mixture of several tubes of third generation Rous sarcoma culture, containing chick embryo but no serum, because of a misconception as to the permissibility of omitting serum not only from primary cultures but from passage tubes.

Chick No.	Injection	Date of death or killing and autopsy finding	
1	1 Cl_1 — 1.5 cc.	5/12	—
	2 Cl_1 — 1.5 cc. + V — 0.5 cc.		+
2	1 Cl_1 — 1.0 cc.	6/1†	—
	2 Cl_1 — 1.0 cc. + V — 0.5 cc.		—
3	1 Cl_1 — 0.5 cc.	5/17	—
	2 Cl_1 — 0.5 cc. + V — 0.5 cc.		+
4	1 Cl_1 — 0.1 cc.	7/17	—
	2 Cl_1 — 0.1 cc. + V — 0.5 cc.		—
5	1 Cl_1 — 0.05 cc.	6/5†	+
	2 Cl_1 — 0.05 cc. + V — 0.5 cc.		—

Chicks 6 to 17—injected according to same protocol with Cl_2 and Cl_2 V. No tumor resulted in any of them.

† Chickens marked thus died on the date shown either from the tumor or from intercurrent disease, mostly extreme emaciation (following coccidiosis?) with occasional instances of chicken paralysis and undetermined causes. Where the symbol is omitted, and it is not stated otherwise, it is signified that the chicken was killed on that date or missing without record subsequently.

The tumors developing at the site of the injection of the mixture in Chicks 1 and 3 are completely offset by the one found in Chick 5

at the site of injection of chloroform filtrate alone in a quantity of only 0.05 cc. and illustrate our main point that not only variation in chickens but some uncontrollable variation at the site of injection itself may determine formation of a tumor. It will be noted that since no serum was added to these virus cultures, no living virus should have been present in these tubes, and consequently, no accelerative action expected.

Experiment May 12, 1926.—Sand filtrate prepared from desiccated tumor. Eight tubes of 10 cc. each divided into four pairs, and chloroform added to each tube, as follows: 1st pair, 0.02 cc. each, 2nd, 0.03, 3rd, 0.04, and 4th, 0.05 cc Water bath half an hour, mixed with capillary, and water bath $2\frac{1}{2}$ hours longer. Each pair of similar tubes was mixed and the chloroform boiled out *in vacuo*. It was noted at time of mixing with capillary that all of the chloroform added to one tube of the first pair had evaporated from the surface, the quantity being too small to form a drop of sufficient size to sink. The preparations are as usual marked Cl_1, Cl_2, Cl_3, and Cl_4.

Virus preparations are as follows:

V_1—4 day primary anaerobic Mouse Sarcoma 180 sterile by smear, but shown later by culture to be contaminated.

V_2—4 day primary anaerobic Rat Sarcoma 10 sterile by smear and culture.

V_3—Uninoculated tube of 10 day chick embryo in Hartley broth, 4 days' anaerobic incubation, sterile.

Chick No.	Injection	Date of death or killing	Result 1	2
18	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. V_1 — 0.5 cc.	6/13†	++	±
19	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. V_1 — 0.5 cc.	6/10	++	+
20	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. V_1 — 0.5 cc.	6/21†	++	+
21	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. V_2 — 0.5 cc.	6/7	+	+
22	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. V_2 — 0.5 cc.	7/23	—	—
23	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. V_2 — 0.5 cc.	6/15†	++	+
24	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. V_3 — 0.5 cc.	6/5	++	++
25	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. V_3 — 0.5 cc.	6/13†	++	++
26	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. V_3 — 0.5 cc.	6/9†	++	++
27	1 Cl_2 — 1.5 cc. 2 Cl_2 — 1.5 cc. V_1 — 0.5 cc.	7/27	—	—
28	1 Cl_2 — 1.0 cc. 2 Cl_2 — 1.0 cc. V_1 — 0.5 cc.	6/16†	+	—
29	1 Cl_2 — 0.5 cc. 2 Cl_2 — 0.5 cc. V_1 — 0.5 cc.	7/17†	++	—
30	1 Cl_2 — 1.5 cc. 2 Cl_2 — 1.5 cc. V_2 — 0.5 cc.	6/21	++	++
31	1 Cl_2 — 1.0 cc. 2 Cl_2 — 1.0 cc. V_2 — 0.5 cc.	5/19†	—	—
32	1 Cl_2 — 0.5 cc. 2 Cl_2 — 0.5 cc. V_2 — 0.5 cc.	6/7†	—	—

Chick No.	Injection		Date of death or killing	Result 1	2
33	1 Cl$_2$ — 1.5 cc.			++	
	2 Cl$_2$ — 1.5 cc.	V$_3$ — 0.5 cc.	6/21†		—
34	1 Cl$_2$ — 1.0 cc.			—	
	2 Cl$_2$ — 1.0 cc.	V$_3$ — 0.5 cc.	6/13†		±
35	1 Cl$_2$ — 0.5 cc.			—	
	2 Cl$_2$ — 0.5 cc.	V$_3$ — 0.5 cc.	8/14		—
36	1 Cl$_3$ — 1.5 cc.			—	
	2 Cl$_3$ — 1.5 cc.	V$_1$ — 0.5 cc.	7/17		—
37	1 Cl$_3$ — 1.0 cc.			—	
	2 Cl$_3$ — 1.0 cc.	V$_1$ — 0.5 cc.	5/28†		—
38	1 Cl$_3$ — 0.5 cc.			—	
	2 Cl$_3$ — 0.5 cc.	V$_1$ — 0.5 cc.	7/17		—
39	1 Cl$_3$ — 1.5 cc.			—	
	2 Cl$_3$ — 1.5 cc.	V$_2$ — 0.5 cc.	8/21		—
40	1 Cl$_3$ — 1.0 cc.			—	
	2 Cl$_3$ — 1.0 cc.	V$_2$ — 0.5 cc.	9/8		—
41	1 Cl$_3$ — 0.5 cc.			—	
	2 Cl$_3$ — 0.5 cc.	V$_2$ — 0.5 cc.	7/4*		—
42	1 Cl$_3$ — 1.5 cc.			—	
	2 Cl$_3$ — 1.5 cc.	V$_3$ — 0.5 cc.	6/2		—
43	1 Cl$_3$ — 1.0 cc.			—	
	2 Cl$_3$ — 1.0 cc.	V$_3$ — 0.5 cc.	9/8		—
44	1 Cl$_3$ — 0.5 cc.			—	
	2 Cl$_3$ — 0.5 cc.	V$_3$ — 0.5 cc.	9/8		—
45	1 Cl$_4$ — 1.5 cc.			—	
	2 Cl$_4$ — 1.5 cc.	V$_1$ — 0.5 cc.	6/9†		—
46	1 Cl$_4$ — 1.0 cc.			—	
	2 Cl$_4$ — 1.0 cc.	V$_1$ — 0.5 cc.	8/14		±
47	1 Cl$_4$ — 0.5 cc.			—	
	2 Cl$_4$ — 0.5 cc.	V$_1$ — 0.5 cc.	7/11†		++

* No tumors at site of injections.   Body cavity full of tumors.

Chick No.	Injection	Date of death or killing	Result 1	2
48	1 Cl_4 — 1.5 cc. 2 Cl_4 — 1.5 cc. V_2 — 0.5 cc.	7/4†	—	—
49	1 Cl_4 — 1.0 cc. 2 Cl_4 — 1.0 cc. V_2 — 0.5 cc.	7/23	—	—
50	1 Cl_4 — 0.5 cc. 2 Cl_4 — 0.5 cc. V_2 — 0.5 cc.	7/23	—	—
51	1 Cl_4 — 1.5 cc. 2 Cl_4 — 1.5 cc. V_1 — 0.5 cc.	6/21†	++	
52	1 Cl_4 — 1.0 cc. 2 Cl_4 — 1.0 cc. V_2 — 0.5 cc.	6/7	—	—
53	1 Cl_4 — 0.5 cc. 2 Cl_4 — 0.5 cc. V_2 — 0.5 cc.	7/23	—	—

The specific factor Cl_1 was obviously insufficiently treated with chloroform since most of that added to one of the tubes was noted to evaporate from the surface during the first half-hour's incubation. Chicks 47 and 51, injected with the specific factor receiving the largest amount of chloroform, are suggestive. However, No. 47 received V_1, a culture later found to be contaminated, and No. 51 received V_2, an uninoculated chick embryo control. It seems justifiable to look on these as entirely accidental. Certainly one should expect a more striking effect on many of the other chickens before accepting these as evidence of a satisfactory experiment.

Experiment May 28, 1926.—Two lots of 35 cc. each of sand filtrate from desiccated tumor treated with 0.07 cc. and 0.14 cc. respectively, of chloroform, mixed at once with pipette, placed in water bath at 37°, and mixed again at the end of half an hour; after a total of 3 hours, chloroform removed *in vacuo;* noted as Cl_1 and Cl_2 respectively.

The following virus preparations used, all 4 day anaerobics:

V_1—Rat Sarcoma 10—sterile on culture.

V_2—Mouse Sarcoma 180—sterile on culture.

V_3—Mouse Carcinoma D Br B—sterile on culture.

V_4—Rat embryo.

V_5—Rat placenta.

Chick No.	Injection	Date of death or killing	Result 1	2
54	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. + V_1 — 0.5 cc.	Missing		
55	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. + V_1 — 0.5 cc.	7/6	++ —*	
56	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8**	— —	
57	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. + V_2 — 0.5 cc.	6/27†	++ —	
58	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. + V_2 — 0.5 cc.	Missing		
59	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	6/16†	— —	
60	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. + V_3 — 0.5 cc.	8/6	— —	
61	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. + V_3 — 0.5 cc.	7/4	— ++	
62	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	6/29	— —	
63	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. + V_4 — 0.5 cc.	6/1†	— —	
64	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. + V_4 — 0.5 cc.	6/25†	— ++	
65	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	7/24†	++ —	
66	1 Cl_1 — 1.5 cc. 2 Cl_1 — 1.5 cc. + V_5 — 0.5 cc.	6/10†	— —	
67	1 Cl_1 — 1.0 cc. 2 Cl_1 — 1.0 cc. + V_5 — 0.5 cc.	7/26†	— ++	
68	1 Cl_1 — 0.5 cc. 2 Cl_1 — 0.5 cc. + V_5 — 0.5 cc.	9/8**	— ±	

* A tumor formed at this site in 3 weeks but had completely retrogressed when the animal was killed.
** Still living.

Chicks 69 to 83 were injected according to the same scheme, with Cl_2 instead of Cl_1. No tumors whatever resulted in these animals.

A large per cent of these chicks died of a coccidial infection too soon to show tumors. Most of the others were in bad condition and a number have died in the course of the summer in an extremely emaciated condition. It is sufficient to point out here again the lack of uniformity of results.

Experiment June 18, 1925.—Desiccated tumor. Cl_1 and Cl_2 contained respectively 0.05 cc. and 0.10 cc. chloroform to 25 cc. filtrate.

V_1—Mouse Carcinoma D Br B culture.

V_2—Primary aerobic Rous sarcoma culture, 8 days old.

V_3—Mouse embryo plus mouse placenta.

V_4—Chick embryo.

V_1, V_3, and V_4 were incubated 4 days in anaerobic jar.

All preparations were sterile on culture.

Chick No.	Injection	Date of death or killing	Result
84	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	8/16	— —
85	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/16	++ ++
86	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	8/10†	++ ++
87	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/23†	++ —
88	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/23†	++ —
89	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	— ++
90	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/7†	— ++
91	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/1†	— —
92	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	— —
93	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	6/29†	— —
94	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	8/16	— —
95	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	8/27	— —

Nos. 96 to 108 done exactly as the above, with Cl_2 instead of Cl_1, were all negative on both sides.

* Still living.

One need only refer to the first seven chickens of this protocol to note the type of variations which occur in these experiments.

Experiment June 27.—Sand filtrate from a fresh tumor was used in this experiment. Four 15 cc. lots, designated Cl_1, Cl_2, Cl_3, Cl_4, were treated as usual with 0.045, 0.09, 0.18, and 0.36 cc. chloroform respectively.

V_1—Jensen rat sarcoma.

V_2—Uninoculated chick embryo.

Both grown 4 days anaerobically and sterile on subculture to blood agar.

Chick No.	Injection	Date of death or killing	Results
109	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/8†	− −
110	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/10†	++ ++
111	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/13†	++ ++
112	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	− −
113	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	++ −
114	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/10†	++ −
115	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/16†	++ ++
116	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/16†	++ ++
117	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/19	++ ++
118	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/15†	++ ++
119	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/10†	− −
120	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	++ ++
121	1 Cl_3 — 1.0 cc. 2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	7/4†	− −

Chick No.	Injection	Date of death or killing	Results
122	1 Cl₄ — 1.0 cc. 2 Cl₂ — 0.5 cc. + V₁ — 0.5 cc.	8/15†	+ −
123	1 Cl₄ — 1.0 cc. 2 Cl₂ — 0.5 cc. + V₁ — 0.5 cc.	7/17†	+ −
124	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₂ — 0.5 cc.	7/31†	++ ++
125	1 Cl₂ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₂ — 0.5 cc.	7/27†	++ −
126	1 Cl₃ — 1.0 cc. 2 Cl₃ — 0.5 cc. + V₂ — 0.5 cc.	8/10†	−* −
127	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₁ — 0.5 cc.	8/26	− ++
128	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₁ — 0.5 cc.	7/1	− −
129	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₁ — 0.5 cc.	9/8**	− −
130	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₂ — 0.5 cc.	8/16	+ −
131	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₂ — 0.5 cc.	• 8/7	− −
132	1 Cl₄ — 1.0 cc. 2 Cl₄ — 0.5 cc. + V₂ — 0.5 cc.	8/3	++ −

* This chicken had previously shown a small tumor at Site 1, which at time of death (extreme emaciation), had retrogressed.

** Still living.

In this experiment it is evident that in Cl₄, a preparation was used which just failed to infect most of the chickens. Active agent was obviously still present, yet there was no evidence of activation by anything present in the virus preparations.

Experiment July 18, 1926.—Sand filtrate from a fresh tumor made as usual.
Cl_1, Cl_2, and Cl_3 contained respectively 0.09, 0.18, and 0.36 cc. chloroform to 15 cc. filtrate.

V_1—Jensen sarcoma primary culture.
V_2—Mouse embryo and placenta.
Both incubated 4 days anaerobically, sterile by subculture.

Chick No.	Injection	Date of death or killing	Results
133	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	— —
134	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/10	++ ±
135	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	— —
136	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/21†	+ —
137	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/29†	++ —
138	1 Cl_1 — 1.0 cc. 2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/16	— —
139	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	— ±
140	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	— —
141	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	— —
142	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	— ±
143	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	— —
144	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	— —

* Still living.

Chick No.	Injection	Date of death or killing	Results
145	1 Cl_3 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	− −
146	1 Cl_3 — 1.0 cc. 2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	+ −
147	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8	− −
148	1 Cl_1 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	− −
149	1 Cl_3 — 1.0 cc. 2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	− −
150	1 Cl_2 — 1.0 cc. 2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	8/10	++ −

Here again there is no evidence of reactivation of chloroform filtrates still containing slightly active agent, by the virus preparations used.

GENERAL DISCUSSION.

If one summarizes the results of the larger experiments, and enumerates the results only of those chickens into which chloroform filtrates were injected, giving less than about 50 per cent control takes, the following result is obtained. 70 chickens injected with specific factor in one side, and with a mixture of specific factor and virus into the opposite side were completely negative. Four were positive on both sides. Fifteen showed a take from the specific factor alone, and twelve from the mixture only. These occasional single takes with control or mixture were interspersed in the same experiments, and the figures would seem to establish definitely that such results in work of this kind are completely without significance. We should like, therefore, to suggest that in the presentation of further studies on this subject, enough consecutive protocols be published to make very clear to the reader the general trend of the experiments, and that to guard even here against accidental variations, plenty of controls be included in each experiment.

If we assume, however, as we must, that all the work done so far

has been based upon amply controlled experiments, is there any way in which the discordant results can be either harmonized or explained? There are four types of findings to be considered. Chloroform filtrate from the Rous sarcoma has been found to be reactivated (1) by nothing (Harkins, Schamberg, Kolmer, and Kast, and the writer); (2) only by virus obtained from tumors (Gye); (3) by extracts of tumor, embryonic tissue, and placenta, *i.e.*, rapidly growing cells (Murphy and Cori); and (4) by extracts of tumors, embryo tissue, and normal liver and kidney (Flu).

In Gye's original paper he does not mention controls of normal tissue. In a later discussion of his work (7), however, he stated that he had controls with media containing embryonic tissue with negative results and subsequent information satisfies us that Dr. Gye has given the attention to this form of control which one would expect from such an experienced worker. Murphy, in his turn, does not mention attempts to use normal adult tissues, such as liver and kidney. One is therefore left in doubt as to whether his results might not harmonize with those of Flu. Obviously a positive experiment of this type must have more weight than a negative, and one cannot help feeling that a sufficiently controlled series of experiments in the hands of those who appear to be able to make the phenomenon work at all, should settle this point. Should the activating principle obtained by Gye from tumor tissue be obtainable also from normal tissues, obviously his hypothesis must be incorrect.

Why should an experiment which is simple in theory, and not highly complicated in practical aspects, lead to three or four different results in as many laboratories? The variable factors entering into the work are three, or at most four, *i.e.*, the tumor itself, the specific factor, the virus, and possibly the strain of chickens used. The last of these can probably be ruled out, since there is pretty general agreement about the susceptibility of Barred Plymouth Rock chickens.

The tumor, however, is a completely unknown element. It is a fact, well recognized by those who have had experience with transplantable mammalian tumors, that a most extreme variation, almost periodic in some cases, is shown by neoplasms in the percentage of takes in susceptible animals. For this, no adequate explanation has been advanced. Moreover, it has been shown by Gye (6) that the

Rous sarcoma may suddenly change its properties and for several generations be not transmissible by filtrates, glycerolated or desiccated tissue, but only with living cells, and then as suddenly regain its usual properties. It is conceivable, therefore, that in many generations of growth in different laboratories, tumors originating from a common source should have so altered their properties along somewhat different lines as to account for marked variability in behaviour in such a method as Gye's. This could be completely ruled out only by an exchange of tumor strains and might be worth checking up.

The specific factor can be *proved* to be satisfactory only if it is *known* that the virus is right, and *vice versa*. In other words, in the case of negative results such as our own, it is almost impossible to say which factor is at fault, or whether both are wrong. For example, does our chloroform, especially purified though it has been, contain a trace of something which destroys the chemical specific factor more rapidly than the virus? If not, then in most of our larger experiments we have had preparations which should have been satisfactory, for a range of destructive action has been shown from obviously too little to a probable excess, through the range between the two, some of which should have represented the correct amount. No such indirect method of control is possible, however, for the viruses. One can only hope to get satisfactory cultures by following as closely as possible the directions set down by Gye. We do not consider here the possibility of checking them by ultra-violet photography according to the method of Barnard (8), because of obvious technical difficulties. Very slight differences in culture media, contamination with other organisms perhaps not recognizable by the usual methods of smear and subculture, or some entirely unrecognized difference in method may have meant that we have never once had a suitable virus preparation. It is worth noting, in this connection, that our cultures were often slightly turbid, although no bacteria could be demonstrated in them. We believe the turbidity to have been due to the acid reaction known to be developed by tumor tissue in the presence of certain sugars. If this takes place in a solution containing tissue juices and serum, one expects a certain amount of clouding or precipitation of protein, and we have attributed the phenomenon, perhaps wrongly, to this cause.

If it should subsequently be shown that Gye is right, we should be

inclined to consider that our virus preparations have been consistently at fault, since error in regard to the other factors seems less likely.

The impossibility of controlling any of the three factors entering into the work of course detracts considerably from the value of purely negative results like our own. But we feel that in a subject as important as this, any evidence bearing upon the problem should be placed as soon as possible at the disposal of others who may be interested.

CONCLUSIONS.

It is obviously impossible to draw definite conclusions as to the significance of the differences between our work and Gye's, and still less, of the differences between Gye's work and that of Murphy and of Flu. We can only say that in a fairly large series of experiments, extending over a period of 12 months, we have had absolutely no indication of the necessity of two factors in the production of the Rous sarcoma. In other words, we have been unable to duplicate either the results of Gye or the modified confirmations of his work by Murphy and Flu. We have shown that uncontrollable local and individual variations may produce results in occasional chicks which simulate satisfactory experiments, but when viewed as a whole, mean nothing. Because of the conflicting nature of results obtained by those who have undertaken to repeat the work, and on account of the difficulty of controlling all factors involved, we do not feel that it may be stated definitely that Gye's theory of the cause of cancer is wrong. On the other hand the theory apparently needs more evidence in its support if it is to receive further serious consideration. It is suggested, in order to untangle the subject as rapidly as possible, that future publications should include sufficient consecutive protocols to make the trend of the experiments obvious to the reader.

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DIET AND TISSUE GROWTH.

IV. The Rate of Compensatory Renal Enlargement after Unilateral Nephrectomy in the White Rat.*

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The enlargement of the surviving kidney after unilateral nephrectomy is a well known and generally accepted fact. On the other hand, the character of this enlargement has been long disputed and has been the subject of many investigations. The literature on this point has been summarized by Arataki (1926, *b*) who, in his own studies on rats, found an increase in the size of both the glomeruli and the tubular systems, an hypertrophy, together with an actual cellular increase of the supporting tissue, an hyperplasia. He also believes that there is an hyperplasia of the constituent cells of the glomeruli and tubules.

On equally important phases of this subject, namely the rate of growth and the dietary factors concerned, there is a striking paucity of data. It is the purpose of this communication to report experimental studies on these questions.

As a result of the metabolic processes the nutritive fluid surrounding the tissues is vastly different both physically and chemically from the food ingested. The composition of this fluid medium of the cells changes comparatively little from day to day in a given species. It has been found possible experimentally to vary the quality of the food ingested within surprisingly wide limits without interfering with the normal functions of the organism. On the other hand, investigations on the effects of extreme or abnormal rations

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* A preliminary report of a portion of this work was presented in the *Proceedings of the Society for Experimental Biology and Medicine* (Moise, T. S., and Smith, A. H., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 561).

have disclosed the fact that there are certain unique dietary demands made by the body if growth and optimal functional activity are to be maintained. Insufficient energy intake, lack of essential amino acids, deficiency in certain inorganic salts and absence of accessory food factors all result in the disturbance of activity of certain organs and, grossly, in inhibition of growth. Recent studies have indicated, however, that when stunting occurs certain of the organ systems show a definite persistence of growth (Winters, Smith and Mendel). Smith and Moise (1924) have shown that injured liver tissue in rats is repaired on diets which are inadequate for growth of the body as a whole. It appears that certain organs are more sensitive to dietary insufficiency than others. The relation of diet to tissue growth is thus an intricate one and its investigation merits extension to other organs and systems.

Nothnagel (1886) early came to the conclusion that compensatory renal enlargement is a function of the composition of the blood. That this phenomenon is caused by some stimulus to function was later shown by Sacerdotti (1896) who, after performing unilateral nephrectomy, observed no increase in size of the remaining kidney when the animal fasted. However, when blood from a dog with total nephrectomy was injected into a normal animal without increasing the blood volume, there was evidence of renal enlargement. Enlarged kidneys have been observed in young rats grown on high protein foods and in mature rats given such diets, when both kidneys were present (Osborne, Mendel, Park and Darrow (1923), Reader and Drummond (1925), Jackson and Riggs (1926)). It appears from these experiments that in intact animals the level of non-protein nitrogen of the blood conditioned either by retention, on the one hand, or by abnormally high level of protein in the ration, on the other, influences directly the enlargement of the kidneys. That the same factor plays a large part in causing compensatory enlargement is shown in the present study.

The rate at which the total amount of renal tissue increases in response to these rather specific changes in blood composition is not only of academic interest but also of practical value. Osborne, Mendel, Park and Winternitz (1925) reported enlargement of the two kidneys in rats after they had been given protein-rich food for 1 week. It is of considerable importance in renal surgery to obtain similar statistics concerning the remaining kidney after nephrectomy. So far as we know such information on experimental animals is not available in the literature Hinman (1923) gives data for the compensatory "hypertrophy" of the right kidney in rats after ligation of the left ureter without removing the kidney. In view of the scarcity of the published data, it seemed desirable to institute an extensive series of experiments on the problem of compensatory renal enlargement after unilateral nephrectomy.

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It is obvious that any practical value of such results does not lie in the importance of the size of the organ *per se* but only in its function as correlated with its size. It has been pointed out by Oliver (1924) that in nephrectomized rabbits the weight of the kidneys is not a true index of the functional activity of the organ because the convoluted tubules and the glomeruli grow at unequal rates and urea is largely excreted in the tubules (Oliver, 1921). Studies of renal function to supplement the present investigation are in progress.

EXPERIMENTAL PART.

Methods.—The plan of the experiment was to remove the right kidney, place the animal immediately on the ration to be used and after a definite time interval to remove the remaining kidney for study. This procedure was carried out with the "standard" food, a diet providing the minimal amount of protein for optimal growth and maintenance and with the "high protein" food, which was unusual in that it contained a high concentration of protein. A third phase involved the study of the degree of compensatory renal enlargement after a constant time but with the animals eating diets containing increasing amounts of protein.

Adult white rats were used to eliminate the growth factor in renal enlargement, since it is well known that the organs increase in size as the body grows. Although a fluctuation in body weight occurred during the periods following nephrectomy, the authors believe that the change in size of the kidneys here recorded is one of practically uncomplicated compensation for the loss of kidney tissue as the result of the initial nephrectomy with the protein content of the diet as the only variable factor. An effort was made to use only male rats but in the early parts of the study non-pregnant females also were employed. None of the animals used had been given other than "stock" diets prior to being used in the present work. Individual variation, the chief difficulty inherent in animal experimentation, must be borne in mind in interpreting the present data. This variation may arise from differences either in heredity or in previous environment, which, in turn, might affect not only the gross reaction of the animal to the diet (efficiency of digestion and absorption) but also the response of the kidney cells to changes in blood composition. An effort was made therefore to use a relatively large number of rats for each experimental test period in order to eliminate as far as possible the uncertain factor of individual variation. It was planned to have twelve animals for each period in the study. In a few cases, accidental death reduced the number while in most instances more than twelve survived. In all, the data on considerably more than 300 rats were used in the final calculations.

In all the experiments diets composed of mixtures of purified foodstuffs were used. Casein furnished the protein, lard provided the fat, raw corn starch was used as a source of carbohydrate and an artificial salt mixture supplied the inorganic requirements. Cod liver oil mixed with the other ingredients and dried yeast fed apart from the food were used as sources of vitamines. In all the experiments the food and fresh tap water were given in unlimited quantity. It is im-

portant to insure an abundant supply of water, for with the protein-rich diets the large quantities of urea resulting from the metabolism of the protein constitute a potent diuretic and the increased volume of urine necessitates augmented fluid intake.   In Table I is given the percentage composition of the various diets employed together with the approximate portion of the energy provided by the constituent foodstuffs.   In planning the rations the aim has been to vary the proportion of protein while the other essential parts of the diet, *i.e.*, the salts and vitamines, remained unchanged.   The caloric value of the diets containing 60 per cent or less of casein is approximately 5.3 Calories per gm. food.   As the casein

TABLE I.

Composition of Experimental Diets.

	"Standard" food		Diet 30		Diet 45		Diet 60		Diet 75		"High protein" food		Diet 90	
	Part of diet	Part of total calories												
	per cent	per cent												
Protein (casein)[1]	18	14	30	23	45	35	60	46	75	60	85	76	90	85
Carbohydrate (raw corn-starch)	51	39	39	30	24	18	9	7						
Fat { lard	22	47	22	47	22	47	22	47	17	40	7	24	7	15
Fat { cod liver oil	5		5		5		5		5		4			
Salt mixture[2]	4		4				4		3		4		3	

300 mg. dried yeast daily given with each of the diets.

[1] A commercial product containing 13 per cent nitrogen.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

is increased above 60 per cent the energy value per gm. food becomes progressively less.

The method of caging used is the same as that heretofore described (Smith, Cowgill and Croll, 1925).

The technique of the operation was as follows: The rat was anesthetized with ether, the hair on the right flank was clipped closely and the area was painted with tincture of iodine.   Under aseptic precautions an incision was made just below and parallel to the right costal margin.   The peritoneum was incised and the kidney was lifted up by passing a small aneurysm needle through the pelvis. The perirenal fat was quickly dissected away, the pedicle ligated *en masse* and the kidney removed.

The wound was sutured in two layers and a thin collodion dressing applied. In the beginning of the experiments there was a small operative mortality from hemorrhage but this was eliminated by more complete dissection of the pedicle and thereafter there were no operative deaths. Wound infection was a negligible factor.

Early in the study the weight of the kidneys was obtained as well as linear measurements of the length, width and thickness. This method was finally discarded as unreliable on account of variations in the measurements of the small soft vascular viscus with slight pressure or with a change in the position of the organ to be measured. Immediately following its removal, the kidney, free of fat and hilic structures, was weighed in a stoppered bottle on an analytical balance. The manipulation was so standardized that the differences in amount of blood lost at various times is believed to have been negligible. After weighing, some of the kidneys were prepared for histological study and others were used for determinations of total solids.

The degree of enlargement was calculated as follows: On account of the change in the body weight of the rats during the period of study, the original (right) kidney weight was compared to that of one of Donaldson's (1924) rats of the same body weight and a factor obtained. The weight (A) of the enlarged kidney of the experimental rat was then multiplied by this factor to enable a direct comparison with Donaldson's figure to be made. From this product was subtracted the weight (B) of one kidney of a Donaldson rat weighing the same as the experimental rat at the end of the period. The difference is the actual enlargement and this divided by the weight (B) gives the enlargement in percentage. In view of the loss in body weight immediately following nephrectomy this method of calculation probably magnifies the degree of enlargement in the early periods, especially on the high protein diets. An example of the method of calculation is given below.[1]

Many measurements of both kidneys in normal rats have shown that they are either of the same weight or that the right is slightly heavier than the left. These observations are confirmed by Arataki (1926, *a*) who states that "on the average between birth and 350 days

	Initial body weight	Final body weight	Original kidney weight	Final kidney weight
	gm.	*gm.*	*gm.*	*gm.*
[1] Average of 12 rats present experiment	287	277	1.126	2.419
Donaldson's corresponding values	286.5	277.1	1.175	1.140

Correction factor $= \dfrac{1.175}{1.126} = 1.04$

Corrected final kidney weight $= 2.419 \times 1.04 = 2.515$ gm.

Difference (enlargement) $= 2.515 - 1.140 = 1.375$ gm.

Degree of enlargement $= \dfrac{1.375}{1.140} \times 100 = 121$ per cent

the right kidney is 2.1 per cent heavier than the left in the male and 2.3 per cent heavier in the female. These differences are slight, but since the right kidney is also more often the heavier, they would appear to represent a real, though small, inequality." This fact introduces an error in some of the experiments in that the calculated percentages of enlargement are too low. However, these differences are so slight in view of the inevitable experimental difficulties involved in such work that they may be disregarded.

TABLE II.

Degree of Gross Enlargement of Remaining Kidneys of Rats Given "Standard" Food.

Interval after nephrectomy	Number of animals	Mean degree of enlargement
days		*per cent*
3	11	5
9	10	14
14	15	16
21	12	24
28	15	17
42	12	28
56	15	23
90	11	35
120	13	44
150	11	48

Results.

Changes in Body Weight.—After nephrectomy there was invariably a loss in weight of the experimental animals. The weight remained below the preoperative level in the group on "standard" food for a period of 2 to 4 weeks, by which time it had returned to the previous value and in many cases increased somewhat as the experiment progressed.

In the group on the "high protein" food the body weight decreased within 3 to 4 weeks following nephrectomy to a new level, often 30 or 40 gm. below the preoperative value, at which it remained throughout the experiment or only slowly rose after a period of 4 or 5 months toward the preoperative level. Concomitant with the initial loss in weight there was observed a decreased consumption of food. As

the body weight of this group of rats became stabilized at this new level, the food intake rose until these rats were eating more gm. of food than those on the "standard" diet but, due to the lower caloric value of the "high protein" food, were actually ingesting less energy (see Smith and Carey, 1923). It appears, therefore, that after the immediate shock of the operation, the rise in nitrogenous waste products in the blood acts as a systemic depressant which is
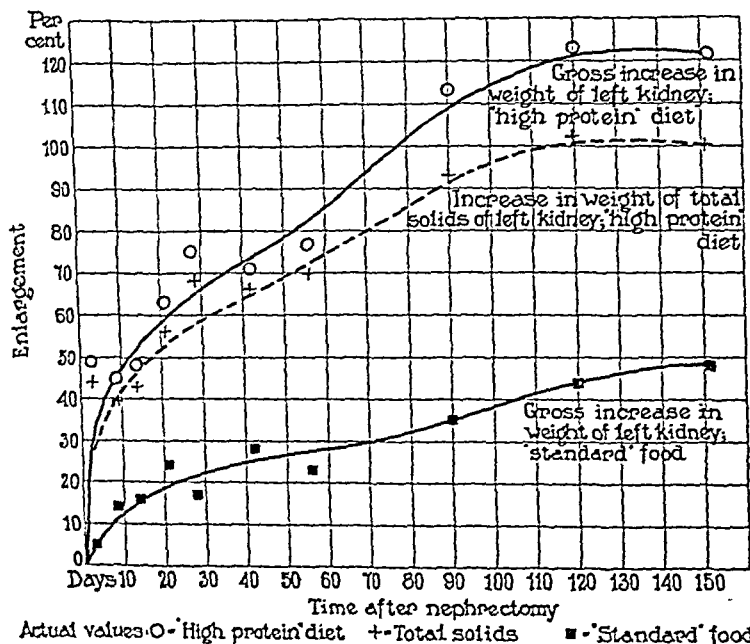
CHART 1. Rate of compensatory enlargement of remaining kidney after unilateral nephrectomy.

reflected in the appetite and body weight of the animal. These observations indicate that in mature rats, physiological well being may be adversely influenced not only by the well known method of decreasing the food intake but also by interfering with the excretion of substances which are primarily waste products. It is more than likely that a definite demonstration of this thesis could be produced in growing rats on protein-rich dietaries.

Rate of Kidney Enlargement.—The results of the study on the rats fed the "standard" food are summarized in Table II and in Chart 1. The individual values for the 3 day period were exceedingly variable, in some of the cases a decrease in size being observed. The recorded value of 5 per cent enlargement after 3 days is therefore only approximately correct. As may be seen from the data there is a rapid increase in the compensatory enlargement of the remaining kidney within the first 3 weeks following unilateral nephrectomy. The left kidney is about one-fifth heavier than the control at this time. The increase after 21 days and until the 120 day period appears to be progressive but at a definitely slower rate (approximately 3 per cent

TABLE III.

Degree of Gross Compensatory Enlargement after 21 Days on Diets of Varying Protein Content.

Casein in diet	Number of animals	Mean degree of enlargement
per cent		*per cent*
18	12	24
30	13	37
45	13	31
60	13	43
75	13	56
85	15	63
90	7	77

in 10 days). The data show that no significant enlargement takes place between the 120th and the 150th days. Hinman (1923) reported that in rats with one ureter ligated, the remaining kidney had reached its maximum compensatory enlargement on the average after 20 to 30 days which does not agree with what is here reported for nephrectomized rats. On the other hand, the data of Addis, Myers and Oliver (1924), when calculated in a way similar to the present method, show that at 106 to 125 days after nephrectomy the remaining kidney of the rabbit shows approximately 32 per cent enlargement, somewhat less than the value indicated for rats in the present study. The details of the diets in either of the above papers are not mentioned.

That the sudden response observed in the early periods is a reaction to the blood composition and the consequent increased demand for renal activity seems probable. Although Bürger and Grauhan (1923) have shown that after nephrectomy in the human there is a pronounced increase in nitrogen excretion, the very important part played by the nature of the diet is shown subsequently in the present study.

The second phase of the experiment demonstrates that dietary protein is at least one of the factors conditioning the compensatory enlargement of the kidney. The plan was altered somewhat for these tests. It was observed in the preceding section that on the
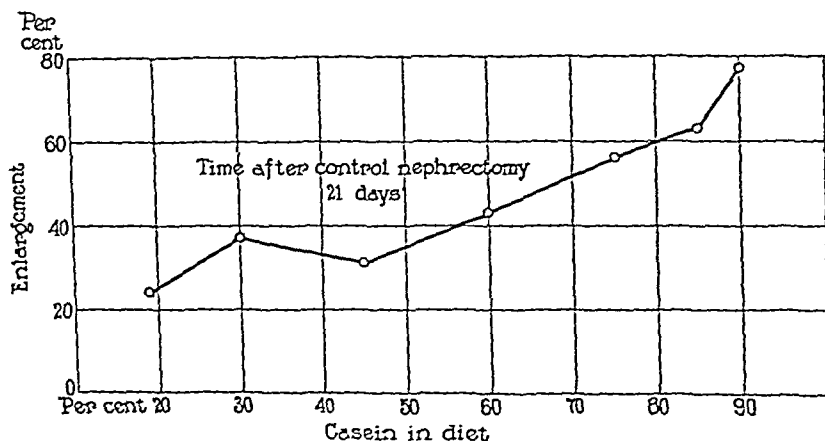
CHART 2. Relation of renal compensatory enlargement to the amount of dietary protein.

"standard" food a marked degree of enlargement could be demonstrated in 21 days. In this second phase the time interval after the first nephrectomy was maintained constant at 21 days and diets with increasing concentrations of protein were used with different groups of rats. Table I shows the rations used.

In Table III are given the data for Chart 2 which shows the degree of enlargement plotted against the per cent of casein in the food. No attempt has been made to draw a "smoothed" curve but, as shown, it is plain that there is almost a direct proportion between the degree of compensatory enlargement and the concentration of dietary protein. In these experiments the catabolic non-

protein nitrogen of Bürger and Grauhan (1923) is constant, since the animals were all treated in the same way and the measurements were made at the same time. Moreover, their published protocols show that in humans the catabolic nitrogen is eliminated within a week. It is obvious, therefore, that the enlargement illustrated by the present data is brought about by some condition depending primarily on the composition of the diet, more specifically, the protein concentration.

What is the maximum compensatory enlargement to which the remaining kidney may be forced under stress resulting from excess dietary protein? To answer this question a series of experiments was planned identical with the first series except that the "high protein" food was used (see Table I). This ration, though extreme, contained all the recognized dietary essentials for growth and maintenance. The data obtained are summarized in Table IV and expressed graphically in Chart 1. The curve showing the rate of enlargement is similar in shape to that of the first series with "standard" food except that the values are much greater. There is the analogous rapid increase in size within the early intervals followed by a slower increase to a maximum. However, where, in the first group on "standard" food, the enlargement at 30 days was about 20 per cent, on the "high protein" diet in a similar length of time the remaining kidney weighed 70 per cent more than the control. The increase progressed at the rate of about 7 per cent in 10 days to the 90th day when the compensatory enlargement was about 113 per cent. From the 90th to the 120th day the rise is much slower and from the 120th day to the 150th day there appears to be no significant increase, the value at 150 days being 121 per cent. It seems probable from these data that even under the stimulus of such a protein-rich diet as here used the maximum enlargement is reached little sooner than on the "standard" food with a relatively low protein content. These combined data show the tremendous physiological reserve possessed by the rat kidney and provide striking evidence for Meltzer's (1906) "factor of safety" in the organism.

The results suggest several questions. Does the increased kidney weight observed after compensatory enlargement result from a *bona fide* increase in renal tissue or is it due to an accumulation of fluid in

the kidney? Many comparisons have been made between the total solids of normal kidneys and of those showing the extreme enlargement on the protein-rich diet. Table V summarizes the data bearing on this point. The average percentage of total solids in the enlarged kidneys in the longer periods, *i.e.*, from 90 to 150 days, is lower than that of normal kidney tissue and the difference is a real one. The total solids per cent of the enlarged kidneys of the 21 to 56 day group is larger but there still is a small though significant variation from the normal. The values for the 3 to 14 day group approaches that of the normal slightly more closely than those of the other two

TABLE IV.

Degree of Compensatory Enlargement of Kidneys of Rats Given "High Protein" Food.

Interval after nephrectomy	Number of animals	Mean degree of enlargement	Mean increase of total solids
days		*per cent*	*per cent*
3	12	49	44
9	13	45	40
14	13	48	43
21	15	63	56
28	14	75	68
42	15	71	64
56	14	77	70
90	10	113	93
120	12	123	102
150	12	121	100

groups. With these data the net tissue enlargement has been calculated for the group on "high protein" food and the results are shown in Chart 1. The degrees of compensatory renal enlargement discussed heretofore in this paper are the gross increase in size, *i.e.*, including the water, the basis upon which other reports in the literature have been made. It is thus obvious that the renal enlargement resulting from extreme conditions of stimulation to functional activity, while a real one, is accompanied by a definite increase in the water content of the kidney and that this is proportional to the time interval during which the animal is given the experimental diet.

Arataki (1926, *b*) has shown that the compensatory enlargement of the remaining kidney is due mainly to the hypertrophy of the glomeruli and tubules. It is possible that the increase in the fluid content of the enlarged kidneys observed in the present experiments is due to its being mechanically retained in the enlarged tubules.

TABLE V.

Total Solids of Kidneys.

Group	Number of rats in groups taken	Degree of enlarge- ment	Mean total solids	Probable error	Significant difference from normal[*] $\dfrac{D}{PEd}$
		per cent	*per cent*	*per cent*	
Normal.................	25		22.3 ± 0.78	±0.101	
"High protein" food[**] 90, 120 and 150 day periods..............	15	113–123	20.2 ± 0.45	±0.078	16.1 Positive
21, 28, 42 and 56 day periods..............	19	64–77	21.4 ± 0.92	±0.15	5.0 Positive
3, 9 and 14 day periods...	14	45–49	21.5 ± 0.56	±0.102	5.7 Positive

[*] D = actual difference between the two averages being compared.

PEd = probable error of the difference.

Difference between the two means is significant when $\dfrac{D}{PEd} > 3$. (See Sherman, H. C., Chemistry of food and nutrition, New York, 3rd edition, 1926, 604.)

[**] The periods were grouped in this manner in order to obtain greater accuracy by using larger numbers of cases. As observed from the chart there is little or no significant difference between the values for gross enlargement within the groups.

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The weights of the heart and liver of some of the rats showing the greatest degree of renal enlargement on the "high protein" diet were compared with similar data for normal animals taken from Donaldson (1924). In no case was an increase in size of either of these organs demonstrable. This observation agrees with that reported by Osborne, Mendel, Park and Darrow (1923), and with many unpublished observations of these investigators.[2]

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[2] Personal communication of Dr. Mendel.

Has the renal function after nephrectomy returned to normal before the final structural equilibrium has been reached or even when this stage is reached? Data bearing on this question have been published by Addis, Myers and Oliver (1924) who used nephrectomized rabbits as the experimental animals. They found that after 106 to 125 days when the *total* renal tissue was 66 per cent of that before nephrectomy the function as measured by the rate of urea excretion was 98 per cent of the normal. The values for rate of compensatory enlargement used by them were those of Hinman (1923) for rats. It seemed necessary to begin new experiments on renal function of rats under the conditions of the present study and the work is now in progress.

Finally, do these extremely large kidneys show evidences of tissue injury? The effect of diets rich in protein on the kidney is at present a favorite theme for debate. The material obtained from the present investigation should afford unique opportunity for such a study, the results of which will soon be published.

Complete protocols of all the experiments have been filed in the archives of the Wistar Institute, where they may be consulted.

SUMMARY.

The rate of compensatory enlargement of the remaining kidney after unilateral nephrectomy has been studied in adult rats fed diets containing various concentrations of protein.

A curve of enlargement on "standard" food (18 per cent casein) shows a rapid initial increase with subsequent slower rise to the 120th day. There is no significant difference between the value at 120 days (44 per cent) and that at 150 days (48 per cent).

A similar series with diets containing increasing concentrations of protein but with a constant time interval (21 days) after nephrectomy shows an increase in the degree of enlargement directly proportional to the protein content of the food. The values vary from 24 per cent with the 18 per cent casein ration to 77 per cent with the 90 per cent casein diet.

A third series shows the enlargement on "high protein" food (85 per cent casein). The values vary from 49 per cent at 3 days to 121

per cent at 150 days. There is no significant difference between the value at 120 days (123 per cent) and that at 150 days (121 per cent).

Determinations of total solids on the experimental kidneys show that the recorded enlargement involves mainly an actual tissue increase.

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A FILTERABLE YEAST-LIKE MICROORGANISM (SCHIZOSACCHAROMYCES FILTRANS, N. SP.).

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PLATE 7.

INTRODUCTION.

The microorganism, the culture and significant qualities of which it is the purpose of this paper to describe briefly, has the interest of a type to those concerned with attempts to cultivate the filterable viruses. From an immediately practical standpoint it may well be regarded as a saprophyte and an accidental contaminant arising in a series of cultures primarily directed toward the study of the filterable virus of hog cholera.

From the beginning almost of our knowledge of filterable viruses as the cause of disease in man and animals it has been a debatable question as to how far filterability is to be regarded as signifying essential invisibility. It has come to be generally recognized that particles in order to pass the coarser earthen filters ordinarily effective for the removal of bacteria must have a smaller diameter of not to exceed one- or possibly two-tenths of a micron. Length, if combined with flexibility, need not be a limiting factor to the same degree. It would doubtless be granted that extensibility within reasonable limits must also be an important factor and that this might be influential in either permitting the passage of somewhat larger particles whose extensibility was great or preventing the passage of very rigid smaller ones. Naturally the state of agglomeration and the condition of the surface, whether adhesive or not, would be additional factors which might determine the actual ability of living particles of smaller dimensions than this to pass a filter. Optically, it is known that particles of about 1/10 micron or less in their longest diameter must appear

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round under even the highest powers of the theoretically perfect microscope. Such particles unless characterized by some peculiar state of agglomeration or by some microchemical reaction must remain indistinguishable from inert particles of the same general size. Unrecognizable they would be essentially invisible.

Within these limits various well known examples may be mentioned. The filterable virus of pleuropneumonia of cattle, long cultivated, is visible without doubt, but admittedly pleomorphic, and this to a degree which combined with the factor of difficult visibility leaves a considerable latitude of uncertainty in the descriptions which have been given of it (1, 2). The virus of *"Hühnerpest"* propagated by Marchoux (3) and by Landsteiner and Berliner (4) was not seen by these authors. More recently described there are the visible and filterable *B. pneumosintes* of Olitsky and Gates (5), the essentially invisible virus of mosaic disease of Olitsky (6), and the likewise optically unrecognizable virus cultivated by Noguchi (7) from the tick *Dermacentor andersoni*. Considering the nature of their growth in culture these microorganisms may probably be minute bacteria. It may well be recalled here that Borrel (8) recognized that certain filterable saprophytic bacteria could be cultivated from water in his locality.

Borrel (9) also gave a brief account of a filterable microorganism from water which he regarded as a protozoan. Schaudinn (10) recognized that certain protozoa might have spirochete-like forms as a stage in a complex life cycle, and, as a suggested application of this postulate, indeed, predicted that yellow fever, of the virus of which it was at that time known that it was transmitted by a mosquito and that it was filterable, would be found to be such a spirochete. The actuality is that the *Leptospira icteroides* of Noguchi (11) is filterable by reason of its inherent properties but there is no evidence of its being a stage in the life cycle of any more complex parasite. These observations of Borrel and Schaudinn served to introduce into the literature of the subject the postulate that some of the filterable viruses might be very small stages in the life cycle of complex parasites, other stages of which might be clearly visible. The thought recurs in the reviews of the subject but concrete examples are so far lacking. Nearest to fulfilling the conditions comes the case of the trypanosomes. Novy (12) described cultures of these protozoa as becoming very small under cer-

tain conditions so that they could pass filters which had been ground thin, and more recently Reich (13) has found that the liver and spleen of animals infected with *Trypanosoma brucei* may frequently yield infectious filtrates. While these observations do relate clearly to protozoan parasites, yet they scarcely apply to the question in hand since there is little or nothing to suggest that the trypanosomes as a group are cyclic in constitution. The culture now in hand, which is in all probability to be classified as a yeast or yeast-like fungus, seems to fulfill quite perfectly the requirements of this very general postulate.

OBSERVATIONAL.

In connection with the study of hog cholera virus it was thought to apply to its cultivation a method developed by Gates (14) during the study of *Bacterium pneumosintes*. Collodion sacs were sealed to a glass tube 1 cm. in diameter and 14 cm. in length (ordinary bacteriological test-tubes with the bottom cut off). These were fitted into larger test-tubes in such a way as to project through and be supported by a tight cotton plug of the latter. The tube with sac attached was also plugged with cotton. Water was put in the inner tube to the height of 10 cm. or more and in the outer tube to cover the collodion, and the whole sterilized in the autoclave. The appliance is then, two tubes, an inner and outer, the fluid in which is separated by a collodion sac 1 cm. in diameter and about 2.5 cm. in length. Either may be separately treated as a bacteriological culture tube.

The experiments in question consisted in replacing the water in the inner tube with mixtures of nutrient bouillon, blood serum of either rabbits or swine, and adding pieces of fresh kidney of either rabbit or swine. A commercial hog cholera virus (unfiltered) was diluted 1/100 with sterile water and the outer tubes of a number of these preparations were inoculated with 1/10 or 2/10 cc. of this. The tubes were then incubated in various ways, aerobically and anaerobically, at room temperature and at 37.5°C., in all possible combinations. A number of these tubes developed growth of ordinary bacteria within a few days and were discarded. Others remained perfectly clear and limpid for weeks and were likewise eventually discarded. A few of the tubes after a week's incubation aerobically at room temperature or 37.5°C. showed a very slight but definite turbidity which remained, but did not increase to the point of sedimentation. Transfers to other similar tubes did not develop. All of these tubes sooner or later developed growth of easily visible microorganisms and were discarded, it being considered that the repeated opening of the rather cumbersome tubes rendered eventual contamination inevitable.

As transfers were made, samples for microscopic examination were reserved in small test-tubes (agglutination tubes) and these were kept on the desk. They were repeatedly examined both in hanging drop and by the application of various

staining methods. There was from the first a reasonable doubt as to whether the particles responsible for the turbidity, which could be clearly seen in hanging drop, might not be living. The reexamination remained a matter of interest for weeks and in the meantime the fluid gradually evaporated. It was several times restored to approximately the original volume with sterile distilled water. Some few days after such an addition of water three tubes in quick succession developed a much greater turbidity with some tendency to the formation of a flocculent precipitate easily redistributed by shaking. This was about 10 weeks after the segregation of the samples and approximately 3 months from the planting of the original cultures. The tubes were now found to contain undoubted microorganisms. So much detail has been given in order to make plain the uncertainties which surround the actual source of the culture.

The slightly turbid samples as segregated from the original tubes and in the succeeding weeks showed moderate numbers of particles, ill defined in optical character. They ranged from those barely visible, through a considerable number just clearly visible, to a small number which may have been 0.5μ in their larger diameters. The smaller particles appeared to be perfectly round; those somewhat larger also were round, and while usually single were sometimes in pairs. The largest of the particles were sometimes round but were often definitely angular suggesting the longitudinal section of an irregular but generally ovoid body. All the particles had active Brownian motion and the larger ones presented a definite halo.

Stained with methylene blue, even with heat or for a long time, and after various methods of fixation, nothing could be seen of these particles. When preparations were fixed in methyl alcohol and stained with full strength carbolfuchsin for from 3 to 5 minutes, or when Giemsa stain was applied, stained particles showing a general correspondence to those seen in the fresh were easily made out, but they presented no qualities sufficiently definite to distinguish them with certainty from precipitated stain or stained detritus. If, as is possible, there was any gradual development of more definite forms such as later appeared suddenly in abundance, it passed unnoticed in spite of repeated examinations.

Examination of hanging drops immediately the sudden increase in turbidity was noticed revealed very considerable numbers of a yeast-like microorganism. This was small as compared to the usual yeast, presenting as characteristic forms more or less round bodies about

1.5μ in diameter associated with longer or shorter structures of the type of abortive mycelia. The number of these was not sufficient to account for the turbidity and they were associated with many much smaller forms. Some of the constituents of the apparent mixture were very actively motile. It seemed impossible at first sight that there could be other than a gross contamination of the fluid but it remained of apparent interest to see whether the smaller forms presented might represent a more active multiplication of the forms previously seen. Suitable preparations were stained and it appeared that there was very little in the material stainable with methylene blue, while carbol-fuchsin and Giemsa stains quite adequately represented the picture presented by fresh preparations. Cultures made on the usual media emphasized the apparent peculiarities in that there was no immediate growth. The material was therefore subjected to a more systematic study with the following final results.

All of those agglutination tubes which showed the sudden increase in turbidity referred to yielded the same culture. The culture grows most consistently on horse blood agar at room temperature. Growth occurs at 37.5°C. but is slower and much less voluminous under these conditions. The addition of sugar to the blood agar neither favors nor interferes with the growth. On this medium even a rather heavy planting shows no change for the first 48 hours. On the 3rd day there may appear an alteration of the surface of the slant and examination with the glass reveals an abundance of very minute, discrete, rather moist colonies. Succeeding days render these visible to the naked eye and at the end of 3 weeks, if the planting has been thin, single, brownish white, mucoid colonies may reach a diameter of 2 to 3 mm. In case the planting has been heavy the covered surface presents a thick creamy mass of the same color. Touched with the wire the colonies are soft, easily spread, but never stringy. They are never soft enough to run down the surface. These appearances are dependent on the medium being freshly prepared, moist, and on the retention of moisture by at least partially closing the tubes. In the case of old medium, or that which has partly dried before the culture is luxuriant, the growth remains flat and dry, the colonies being small and discrete. Intermediate between these extreme alternatives there develop a few mucoid colonies on a surface generally covered with flat, dry,

discrete ones. Such cultures suggest a mixture, but transfer from any part of them to favorable medium restores the described luxuriant growth.

The blood in the agar shows no characteristic changes as growth develops. The condensation water of these blood agar tubes remains clear but gradually develops a heavy sediment. Scraped into distilled water, normal saline solution, or nutrient bouillon, the growth from the blood agar slant forms a homogeneous suspension with the greatest ease. This partly sediments in 2 or 3 days but is resuspended by slight shaking. The appearance (except color) and consistency of the fully devloped culture on blood agar resembles that of a luxuriant culture of ordinary yeast on the same medium.

The culture may be said to grow well on all of the usual culture media but the volume of growth attained on the other solid preparations used does not equal that on blood agar. Growth on media other than blood agar is also capricious in marked degree; that is, unless the culture from which the transfer is made is at the height of its vigor, the transfer is apt to fail or the resultant growth be very uneven. In bouillon with or without added sugar growth results in a flocculent or granular sediment tending to cling to the sides of the tube until dislodged by light shaking. There is sometimes a light ring at the top suggesting that growth takes place here and that the sediment results from the fall of this imperfect scum. When serum is added to the bouillon growth starts in the same way but the resulting sediment is much larger in amount and less granular. After several weeks there gradually develops a diffuse, heavy clouding throughout the fluid above the sediment reaching to the top and this clears but slowly over several weeks more.

Planted in a semisolid medium containing reduced amounts of bouillon constituents and with blood serum added (Noguchi's leptospira medium), heavy growth develops on the surface and in the upper few millimeters but does not extend below this.

Neither acid nor gas is developed in fermented bouillon to which Andrade's indicator and various sugars are added. Dextrose, lactose, saccharose, maltose, and mannitol have been tested. In the fermentation tube growth is confined to the open arm.

Growth on plain agar has always been scanty; on glycerol agar it

has sometimes been scanty and sometimes quite heavy, particularly in the lower part of the tube. On glycerol agar the growth has sometimes acquired a pale orange-yellow color with age.

Gelatin is not liquefied.

Litmus milk is not changed.

The microorganism has now been under observation for over a year. The growth characteristics as described have remained constant. The culture has been repeatedly plated and has been and is still apparently pure. Microscopically the culture has changed in that certain forms which were prominent in the early cultures are now more rarely seen. There was in the beginning a greater tendency than now for changes in the medium employed to result in a temporary predominance of particular morphological forms. The following description of the morphology of the culture is a composite based on the total experience with it.

Transfer of culture material which has aged to any medium or of a culture at the height of activity to an unfavorable medium results in a culture which for the first few days consists chiefly of what are at first sight simple micrococci of about 1.5μ diameter. These may be single and perfectly round; more frequently they are clumped in dense masses in which the individual cocci are only apparent at the edges; sometimes they are in pairs definitely lanceolated (Figs. 5 and 6). Cultures in plain bouillon, on plain agar or on blood agar somewhat dry when the plant is made, may develop no other forms than these. The micrococci are mostly stained rather faintly with methylene blue but some individuals are intensely stained. With carbolfuchsin or with Giemsa's stain they stain intensely. Neither in the fresh nor in the stained condition do the cocci exhibit any evidence of structure. As the cultures age the masses of cocci examined in the fresh state assume a definitely granular appearance. They now stain even less well with methylene blue. With Giemsa or fuchsin the granular condition is very evident, the particles being irregularly massed, interspersed with much poorly staining material, and varying in size from that of the original coccus to about 0.1μ. Every effort has been made to secure preparations which might exhibit some regularity of transformation of the cocci into the smaller granules, but without success. This might be but the rather usual granular transformation in old bacterial cultures but for the following facts.

If such a granular culture, or one consisting chiefly of cocci before they have changed, is washed into sterile distilled water and allowed to stand for 72 hours it is found to have changed completely. The masses of round cocci are no longer found in any considerable number and single round cocci are also rare. Ovoid cocci, single and in lanceolated pairs, of the general size of the original cocci, are now quite common and there are numerous forms of the same length (1.5μ) but thinner, so that they appear as definite bacilli (Fig. 7), and even more numerous are still smaller bacillary forms definitely grading down to the smallest visible ones (Fig. 10). Especially interesting are numerous instances in which the thinner bacillary forms appear as branches or buds from the ovoid cocci or the lanceolated pairs (Figs. 8, 9, 11, 12). Many of these definitely bacillary forms are very actively motile. They may exhibit this motility while attached to the larger forms and impart to the latter a very active movement although less rapid than that of the detached bacilli. With the dark-field microscope the very smallest bacillary forms can be recognized as frequently motile. The round or ovoid cocci are never motile in the absence of one of these recognizable buds or branches. The bacillary forms stain not at all with methylene blue and much less readily than do the coccoid forms with carbolfuchsin or Giemsa's method.

This complex result following the suspension of the coccoid culture type in distilled water is an essential restoration of the picture presented by the original samples after they had acquired a marked turbidity, as described in previous paragraphs. The same result is also spontaneously developed in the cultures, being characteristic of those colonies or masses which develop pronounced mucoid characters as likewise described above. There is a difference in that in the cultures the coccoid type is never so completely submerged as is often the case in the water suspension. A further difference is that the suspension of the culture in distilled water usually presents appreciable numbers of the larger yeast-like forms (Figs. 1 and 2) seen in the original material, while under cultural conditions favoring the mucoid transformation and the development of the motile bacillary forms the larger yeast forms are seldom or never seen.

The yeast-like forms appear in abundance under certain conditions which have not been brought under control. They have been some-

times, but are not usually, abundant in the condensation water of blood agar transplants during the first day or two before there is any visible growth. They have also been abundant and persistent in some of the glycerol agar cultures. Examined in fresh preparations when abundant these forms present the widest variety of shape. They are all resolved, however, into a coccus-like body, or clump of such, with a projected mycelial-like structure of variable length, width, and conformation. The mycelial projection frequently exhibits irregular contractions in the fresh condition, and usually does so when fixed and stained. The illustrations (Figs. 1 and 2) are from preparations left overstained. If differentiated the irregularities are emphasized and the projections are often coarsely granular, suggesting a disintegration into coccoid bodies. In the fresh, the mycelia are not granular and there is no suggestion of such a break-up. In the condensation water of blood agar cultures before visible growth appears there are frequently found clumps of coccoid bodies with a fringe of bacillary projections (Fig. 4).

As wholly aberrant there are rarely seen perfectly round bodies of larger size (5–6μ diameter). These in fresh preparation are homogeneous and thin walled, presenting no suggestion of a double contour or any heavier membrane. There are also seen rarely in stained preparations homogeneous, rather densely staining bodies of similar size and conformation. In a very few instances these have shown a suggestion of division into four ovoid bodies corresponding in size to the more usual coccoid forms.

The extreme pleomorphism presented by the original watery suspension and the earliest cultures naturally suggested a mixture. It was hoped to recover the smaller forms by filtration. The following experiment was done as a repetition of imperfectly executed preliminary trials.

Eight new Berkefeld filters, grade N, were prepared in the usual way. A number of cultures on blood agar 2 weeks old were suspended in distilled water and stood at room temperature for 5 days with frequent light shaking. The total quantity of suspension was about 100 cc. There was then added to this an equal amount of a heavy suspension of *Bacillus abortus* (Bang) made from an old laboratory culture capable of vigorous growth. This mixed suspension was immediately passed through the filters, 20 cc. through

each of the eight. The filtration was forced by the house vacuum and occupied about 10 minutes each. Each filtrate was divided immediately into two equal portions. One portion was planted on plain agar, 1 cc. per tube, the tubes sealed with wax, and incubated at 37.5°C. for 10 days as a test for *B. abortus*. The other portion was planted, 1 cc. per tube, on horse blood agar and kept at room temperature.

The experiment resulted as follows. Two of the filters passed *B. abortus* as well as the microorganism in question and are considered defective. Of the six shown to be efficient in removing *B. abortus*, the microorganism in question passed through three. Growth from the filtrates was slow, only developing after 2 to 3 weeks, and in only one case did all the tubes develop growth. In the other two cases about half the tubes developed. The growth which did develop was normal for the complete culture. All the forms which have been described were eventually observed in the first cultures from the filtrate. The small colonies consisting of cocci chiefly were the first to appear, but the mucoid transformation was prompt in all cases and was associated with the appearance of the motile bacillary forms. The yeast-like forms appeared when these first filtrate cultures were suspended in distilled water. This experiment was carried out when the microorganism had been under active cultivation for about 4 months.

In view of the possible origin of the culture in the virus blood of hog cholera its virulence for swine has been repeatedly tested. It does not produce cholera nor did the experiments develop any other evidence of pathogenicity.

DISCUSSION.

The culture described fulfills the condition postulated in the introduction. That is, we have to do with a pleomorphic microorganism certain forms of which are within the range of ordinary microscopic visibility, other forms at the limits of visibility, would, taken alone, be essentially invisible. Under certain conditions forms capable of regenerating the complete culture are filterable through Berkefeld N filters.

It follows as a practical consequence of this that in dealing with filterable virus material as the subject of cultivation experiments growths which are within the range of ordinary visibility may not be disregarded or discarded as inconsequential on this count alone.

The consideration of the systematic position of this microorganism is of considerable secondary interest. Because of the appearance of the larger forms referred to, the general character of the luxuriant growth, and the evident complexity of the cultural forms, I have considered that it was probably to be classified as a yeast or yeast-like fungus. The predominant type of multiplication in the earlier stages of all cultures and in all stages when cultural conditions are rather unfavorable is by fission. If, therefore, the assignment of the culture to the yeasts were accepted, it would fall naturally into the group of schizosaccharomyces.

The schizosaccharomyces as described by Guilliermond (15) seem to comprise two distinct groups at the present time. One of these is made up of a number of well known cultures closely related in fermentational activity, size, and general morphology to the yeasts whose direct multiplication is by budding. The other group comprises a number of microorganisms found in the organs of insects. They have been generally accepted as yeasts on morphological grounds but have not been cultivated. They are of smaller size on the whole than the first group and their systematic position would still seem to be open to some question. It is interesting that two of these, found in *Chermetis strobilobii* Kalt. and *Chermetis abietis* L., as described and classified by Sulc (16), are the smallest of the yeasts (length $1–2\mu$) and in this respect are comparable to the fission forms of the culture in hand. Considered to be a yeast the culture in hand appears to be more closely allied to these insect forms than to any other.

As an alternative to this assignment the microorganism above described might be thought of as a pleomorphic bacterium. The curious phenomenon of a non-motile micrococcus with motile bacillary branches would be presented, together with extreme changes in size in both the morphological series. The larger forms which have been thought of as yeasts might be reconciled with this view as involution forms. On this alternative the culture would have many points of resemblance to the group *B. radiobacter* of the root nodules of legumes and to *B. azotobacter* of the soil. With these bacteria extreme pleomorphism prevails and very small forms occur. Löhnis' (17, 18) attempts to recover the culture from filtrates were apparently unsuccessful in the case of *B. azotobacter*.

A second alternative has been constantly in mind, namely, that the culture may really be a mixture. This has seemed less probable as the properties of the culture have been developed. It involves the conception of at least two microorganisms both filterable and living in such close association that they are inseparable by plating methods. It need only be pointed out that even accepting this alternative it in no way diminishes the significance of the culture as a type of filterable microorganism presenting easily visible forms and readily cultivatable.

It may further be suggested that if this microorganism is a yeast the basic forms, small as they are, should show more evidence of definite internal structure than is the case.

While suggesting such restrictions and alternative interpretations as have arisen during the study of the culture, my inclination is to regard the microorganism as a fission yeast further characterized by its filterability and therefore designated provisionally as *Schizosaccharomyces filtrans*.

SUMMARY.

A microorganism cultivated in the course of experiments with the virus of hog cholera shows the following points of interest.

It is pleomorphic in extreme degree.

Among the forms are coccoid and bacillary bodies at the lower limits of visibility.

The culture can be completely regenerated after filtration through some Berkefeld N filters although these retain *Bacillus abortus* (Bang).

The culture is carried forward by multiplication of forms of easy visibility (1.5μ).

Associated with these at times are still larger forms bearing a striking general resemblance to those yeasts which develop abortive mycelia.

Motile bacillary forms appear to arise as buds or branches from round or ovoid cocci.

While cognizance is taken of possible alternatives the microorganism is provisionally classified with the yeasts and recognizing its filterability is named *Schizosaccharomyces filtrans*.

The culture is not pathogenic for swine.

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EXPLANATION OF PLATE 7.

FIGS. 1, 2, and 3.   From 14 day culture on glycerol agar.   Giemsa stain without differentiation.   × 1620.

FIG. 4. From blood agar culture suspended in distilled water.   Giemsa stain without differentiation.   × 1620.

FIG. 5. From 2 day culture on blood agar.   Carbolfuchsin stain.   × 1620.

FIG. 6. Same culture as Fig. 5, stained with Giemsa's method.   × 1620.

FIGS. 7, 8, 9, 10, 11, and 12.   From suspensions of blood agar cultures in distilled water.   Stained by Giemsa's method.   × 1620.

Note.

The bacillary and branched or budded forms as shown in Figs. 7 to 12 are apt to be very actively motile.

The independent division of the bacillary forms is strongly suggested in Fig. 7.

The origin of the bacillary from the coccoid forms is strongly suggested in Figs. 11 and 12, where the suspensions were active and the cocci free, as well as in Fig. 4 from a suspension of an old culture where the cocci remained in masses.

The granular transformation of the coccoid forms is shown in Figs. 5 and 6.

The yeast-like forms are shown in Figs. 1 and 2, while Fig. 3 from the same preparation shows how close may be the relation between these and the coccoid forms of Figs. 11 and 12.

An attempt to construct a complete developmental cycle on the basis of these suggestions seems to be unwarranted because of an insufficient understanding of what happens among the smallest forms of Figs. 5 and following.
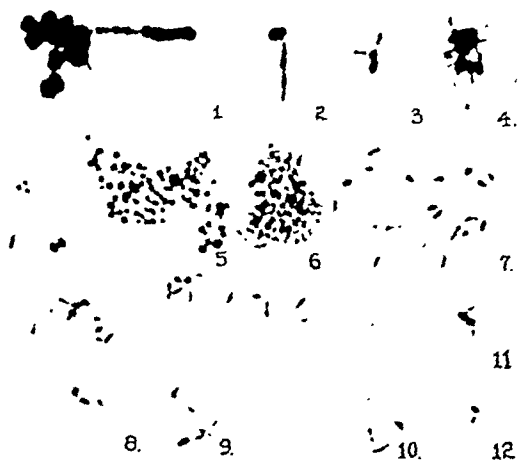
THE HISTOLOGICAL EXPRESSION OF THE NATURAL RESISTANCE OF RABBITS TO INFECTION WITH HUMAN AND BOVINE TYPE TUBERCLE BACILLI.

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The separation of mammalian tubercle bacilli into two types, designated respectively the human and the bovine in accordance with their usual derivation, as first made by Theobald Smith depended primarily on a constant difference in virulence. The recently isolated cultures of bovine type in minimal doses cause a progressive, usually fatal tuberculosis when reinoculated on young cattle or on rabbits. In equivalent doses cultures of the human type cause only local, non-progressing lesions in these animals, lesions which frequently heal entirely. Smith later described differences in the progressive changes in the reaction curve when the type cultures are grown on glycerol broth of appropriate constitution, and also other less definitely measurable cultural distinctions were clearly drawn.

The foregoing statements of fact may be paraphrased with the reaction of the animal as a primary consideration. We would then say that cattle and rabbits have a high degree of natural immunity or resistance against infection with tubercle bacilli of the human type and are very susceptible to infection with those of bovine type. This natural immunity toward bacilli of the human type is by no means absolute. In the case of the rabbit the kidneys frequently become the seat of a slowly progressive local disease which eventually destroys their capacity to function and leads to the death of the animal. At times also quite extensive loci of disease develop in the lungs. The restraint is, however, sufficient to prevent a generalized spread of the infection, and death when it occurs is caused by interference with some local function.

The nature of this natural immunity is quite unknown and is a subject of considerable interest.   It is known that the injection of human tubercle bacilli intravenously does at times lead to the formation of miliary tubercles in the lungs and that these must subsequently be reabsorbed to account for the generally normal condition of these organs when the animals are killed 2 or 3 months after infection.   The resistance does not therefore depend on a total failure of the tissues to react to the presence of the bacillus.   Nor can the natural resistance have all the qualities of the acquired immunity possessed against reinfection in greater or less degree by all tuberculous animals which have been infected for several weeks.   It is generally considered that hypersensitiveness as manifested by a hyperactivity of exudative and destructive processes is in considerable measure responsible for this induced partially immune condition.   The hypersensitiveness is lacking, of course, in the normal animal and takes as a matter of fact 2 or 3 weeks to develop its effectiveness.   It must be assumed that this feature is lacking in the early days of a primary infection and can be at most a reinforcing factor in any expression of natural resistance. Beyond these two points, the certainty of some positive reaction to the human bacilli and the certain absence of the hypersensitive factors in the earlier stages at least of this reaction, present knowledge fails us.

As a preliminary to a further study of this matter we have carried out a comparison of the reaction of the lung of the rabbit to the two types of bacilli using histological methods.   The purpose of the present paper is to report these observations.

Krause[1] outlines a somewhat similar study as being then in progress in his laboratory.   His comments, directed wholly to problems related to the localization of the infection, make no reference to the comparative aspects of the study and do not suggest any particular reference to the question of immunity.

<center>EXPERIMENTAL PART.</center>

Fifteen rabbits of approximately the same weight and age were injected intravenously with 1 mg. of a culture of living human tubercle bacilli, and the same

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[1] Krause, A. K., *Am. Rev. Tuberc.*, 1922–23, vi, 1.

number with 1 mg. of a living bovine strain.   At 2 day intervals an animal of each series was killed, tissues being preserved in formalin and Zenker's fluid.   Sections were cut in paraffin.   Staining was done with the eosin-methylene blue method after Mallory and Wright and for tubercle bacilli by the Ziehl-Neelsen method.   A description of the conditions found in representative individuals of each series on microscopic examination follows.   The gross appearances were not informing.

The human type culture (No. 7156) used was a typical one isolated from sputum at the Henry Phipps Institute of the University of Pennsylvania in the fall of 1922.   It has been cultivated on glycerol agar with monthly transfers.   The virulence for guinea pigs is normal for the type.

The bovine type culture (Bovine xiv) was isolated from a cervical abscess occurring in a 9 months old calf in May of 1919.   The location and character of the lesions were rather atypical.   The material was injected into guinea pigs and cultures isolated from them on egg medium in the usual way.

After four monthly transplantations on the egg medium the culture was placed on glycerol agar and has since been maintained on this medium.   The growth characteristics and virulence for guinea pigs and rabbits are characteristic for the type.

Rabbit 1.—Human bacilli injected.   2 days.   There is much thickening of the interalveolar septa very irregularly distributed.   This is partly due to a very intense capillary congestion and stasis, in part to an exudation of mononuclear and polynuclear leucocytes.   The mononuclear cells are of varying size and conformation, chiefly large cells of "endothelial" type.   Small lymphocytes are rare, and definite phagocytic macrophages are not seen.   The polynuclear cells are chiefly of eosinophilic type, showing large granules rather indistinct in outline with a tendency to fusion.   These cells are found especially in foci about the smaller arterioles, sometimes in small collections in capillaries and venules.   The alveoli are generally empty of either cellular or serous exudation.   Occasionally one is found to be filled with an exudate chiefly made up of large mononuclear cells.   The smaller bronchial branches are frequently filled with similar cells showing that while, as measured by the alveoli affected, the exudation to the surface is slight, yet *"in toto"* it is significant.

The peribronchial lymphoid tissue is not large in amount.   About the smaller divisions it shows some congestion in the peripheral zone and a slight increase of large mononuclear cells toward the center of the mass.   A small amount of cellular debris is found in this region also.   In connection with the larger bronchial branches lymphoid nodules are found between the cartilaginous plates and the mucosa, which are swollen, have broken into and in one instance through the mucosa.

A particular search for mitotic figures in those places where large mononuclear cells are collected in foci, both in the interalveolar septa and in the lymphoid tissue, failed to reveal any.

Tubercle bacilli were found in small numbers in the peribronchial lymphoid tissue. They appeared to be extracellular. None were found in other places.

Rabbit 4.—Human tubercle bacilli injected. 8 days. The interalveolar septa are much thickened and folded decreasing appreciably and generally the area of the alveoli. The thickening is due chiefly to an exudation of large mononuclear leucocytes. A moderate number of polymorphonuclear leucocytes are found. There is no congestion.

At widely scattered points the thickening of the septa is more massive and one or more alveoli are obliterated. In a few instances at these points there is organization into definite tubercles. In these cases the central cells are mononuclears, more uniform in character than in the exudate generally. In some cases they are of moderate size with basophilic cytoplasm; in others they are larger and both cytoplasm and nuclei stain rather faintly. None show any intense staining with eosin or other evidence of necrosis.

Eosinophil leucocytes are found occasionally in the exudate generally. They are very numerous in the lymphoid tissue about the arteries and arterioles. In one instance an arteriole is thrombosed, the cells of the thrombus being large mononuclears and eosinophils. The eosinophils are found in the muscular coat of this vessel as well as massed in its neighborhood.

Mitotic figures occur occasionally in the large mononuclear cells of the exudate generally. They are found in moderate numbers in the lymphocytes of the peribronchial lymphatic tissue.

Tubercle bacilli are found in moderate numbers in those portions of the interalveolar septa where the formation of tubercles is evident or suggested, but not elsewhere.

Rabbit 7.—Human bacilli injected. 14 days. The general description given of Rabbit 4, the 8th day animal, might well be applied to this animal. The following points of difference are to be noted. The tubercles are on the whole somewhat larger and some of them have a definite peripheral zone of lymphocytes. Tubercle bacilli are found in smaller numbers than in the 8th day animal, and like that one, only in the more central portions of the tubercles. The proliferative process, as evidenced by mitotic figures in large mononuclear cells in the interalveolar septa and in the tubercles, is still active. Eosinophil leucocytes are found but in much smaller numbers than previously.

Rabbit 11.—Human bacilli injected. 22 days. In the gross, there are minute, translucent, non-caseous tubercles scattered over the surface of the lungs. The organ is not definitely more firm to the touch than normal. In general the microscopic picture presented by this animal closely resembles that of the 8th and 14th day ones in this series. The interalveolar septa are markedly thickened, perhaps slightly less so than in the preceding animals. Mitotic figures are not found in them.

The tubercles are slightly more numerous in this animal. They are always single. The centers of the larger ones are almost unstained when viewed with

the low power and have a glassy appearance in some places. The high powers show the nuclei faintly outlined, the cytoplasm hyaline, the whole having taken very little stain. The outlines of individual cells can seldom be made out, small groups being fused and the mass apparently cracked and channeled (perhaps due to fixation shrinkage). The hyalinization process is found to extend in streaks along columns of cells to the periphery of the tubercle in numerous places. This is not accompanied by any crowding of the remaining cells, the consequence being that the whole tubercle is appreciably less in its content of well preserved cells than in the earlier animals. The well preserved cells are mostly large mononuclears. Occasional large phagocytes of the macrophage type are seen. There is a small amount of free cellular debris.

The lymphoid tissue is not prominent. One peribronchial lymphoid mass contains a definite tubercle which is hyalinized as just described. In other places the lymphoid tissue shows aggregates of large mononuclear cells.

Mitotic figures are found in large mononuclear cells in the peripheral portions of the tubercles in small numbers.

Eosinophils and amphophil polynuclear leucocytes are found scattered singly in the septa, in the peripheral zones of the tubercles, and in the lymphoid aggregates. Masses or clumps of these cells are not found.

Tubercle bacilli are found in very small numbers in the centers of the tubercles.

Rabbit 15.—Human bacilli injected. 30 days. The interalveolar septa in the subpleural regions are definitely thickened but in a very irregular way. In small patches this thickening is sufficient to obliterate alveolar spaces, in others it amounts to but the presence of an extra layer of mononuclear cells in the septum. The patches are rather definitely most marked beneath the pleura and in the regions about the vascular trunks fading through the less thickened septa to normal lung tissue in the midzone. Definite tubercles are found both in the subpleural region and in the region about the main vascular trunks. In the less thickened septa there are found small collections of large mononuclear cells more or less interspersed with lymphocytes. Occasional giant cells quite isolated from other cell collections are also found in this midregion.

The peribronchial and perivascular lymphoid tissue is scanty.

The tubercles, some of them large, consist almost entirely of large mononuclear cells. There is no definite demarcation into zones except for the suggestion of an imperfect capsular layer one or two cells thick. The cells are chiefly large mononuclears. Some of them are hyaline both as to nucleus and cytoplasm, staining very faintly. Others, perhaps the majority, are of normal appearance. The normal and abnormal cells are intermixed with no regular pattern. Occasional lymphocytes and a minimal amount of cellular debris are found among the mononuclear cells.

No mitotic figures were found and no tubercle bacilli.

Rabbit 16.—Bovine bacilli injected. 2 days. There is a very marked general thickening of the alveolar walls by an exudation consisting of large and small

mononuclear and polymorphonuclear cells. Many of the latter are of eosino-phil type. The thickening of the septa is sufficient in many places to almost obliterate the alveoli by compression although the alveolar wall remains clearly defined and the alveoli generally are quite free of exudate. There is no marked congestion either generally or focally. The mononuclear cells of the exudate are on the whole rather large and faintly staining. The nuclei tend to take a violet tinge with eosin-methylene blue. There are scattered, rather large aggregations of mononuclear cells, with a moderate amount of cytoplasm, which retain a rather intense, clear blue with the stain. An occasional mitotic figure is found in the interalveolar septa, pertaining to cells of the large mononuclear series.

The lymphoid tissue about the bronchial vessels is greatly increased in amount chiefly by an increase in the size of the aggregates. Lymphoid cells undergoing mitosis are found in very large numbers in the peripheral zone of the nodules. The lymphoid tissue shows a scattering infiltration with large mononuclear and polymorphonuclear leucocytes.

Tubercle bacilli, taking the sections generally, are found in but small numbers and then in the interalveolar septa; none were found in the lymphoid tissue. In a few focal points—those previously described where the septa were most swollen —collections of large numbers of bacilli, single and in clusters were found. The arrangement of the clusters was such as to suggest the intracellular position of the bacilli.

Rabbit 19.—Bovine bacilli injected. 8 days. The interalveolar septa are generally much thickened and folded due to the excessive cellular content con-sisting of large mononuclear cells chiefly. Many polymorphonuclear cells are also found, eosinophils being more numerous than amphophils. Over considerable areas the thickening of the septa is of such extent as to nearly or quite obliterate the alveoli, the wall of the latter often remaining intact. In many places there is a very definite organization of the cellular content of the septa into tubercles and these are in various stages. Some are little more than collections of large mononuclear cells with a tendency to basophilic staining associated with a few amphophilic and eosinophilic polymorphonuclear leucocytes. In others similar groups of cells are partly surrounded by and merged in the periphery with lym-phocytes. In a few places the mononuclear cells are massed closely and show decreased staining intensity. There is no definite necrosis. The tubercles are more numerous and somewhat further advanced than in the "human" animal of the same date.

The periarterial lymphoid tissue shows masses of eosinophils.

The peribronchial lymphoidal aggregates are of large size. In some instances their central portions almost to the periphery are occupied by large mononuclear cells. In these instances the central portions also contain small foci of lympho-cytes, some polymorphonuclear leucocytes, and a moderate amount of cellular debris. In some instances also the submucosa and mucosa of the adjacent bron-chial vessel are infiltrated with lymphocytes even to the surface.

Mitotic figures are abundant in large mononuclear cells in the interalveolar septa; in large mononuclear cells in the tubercles; in large mononuclear cells in the central portions of the lymphoid aggregates in those instances in which these cells are prominent there; and in the lymphocytes in the lymphoid tissue generally.

Tubercle bacilli, single and in clusters, are abundant in the central portions of the more advanced tubercles. They are found single and in small numbers in the peripheral portions of the tubercles among the lymphoid cells and in the peribronchial lymphoid tissue.

Rabbit 22.—Bovine bacilli injected. 14 days. In the gross, the pleural surfaces of all lobes are marked with minute, glistening, translucent, raised tubercles, giving the lungs a granular appearance. Some of these tubercles have coalesced. Organ is firm.

The interalveolar septa generally are somewhat less thickened than in the previous rabbits of the bovine series. Mitotic figures are no longer to be found in any number in the septa when these are definitely removed from well defined tubercles.

The tubercles are much more numerous than in the 8th day animal, the addition in numbers being apparently due in large part to the organization of new, small tubercles in contiguity with the older ones. The lines of demarcation while generally rather distinct are not always so. The larger tubercles have undergone a very definite central necrosis.

Eosinophilia is pronounced in the peripheral regions of the tubercles, in the interalveolar septa, and particularly in the periarterial lymphoid tissue.

Proliferation as evidenced by mitotic figures is still in evidence in the large mononuclear cells in the peripheral portions of the smaller tubercles and in similar cells in some of the lymphoid aggregates. No mitoses are found among the lymphocytes.

The lymphoid tissue shows some well defined tubercles. The lymphocytes are quite generally replaced by large mononuclear cells.

Tubercle bacilli are very abundant in the tubercles particularly in the central portions of the older ones. They are also abundant in those areas of the lymphoid tissue where the large mononuclear type of cell predominates.

Rabbit 26.—Bovine bacilli injected. 22 days. In the gross, the lungs are much enlarged, very firm to touch, and granular. The surface is heavily marked with translucent miliary tubercles most of which have gray centers.

Microscopically, the prevailing picture is that of conglomerate miliary tubercles, the majority of which show central necrosis. These occupy fully two-thirds of the lung. Between some of the tubercles there are large alveolar spaces containing a little exudate and cellular debris, while similar ones are entirely empty. The interalveolar septa where not in contact with tubercles are but slightly thickened.

The peribronchial and periarterial lymphoid tissue is involved in the tuberculous process which here is continuous with that in the adjacent lung structure so

that the lymphoid structure is no longer demarcated or clearly recognizable. Small collections of lymphocytes are scattered here and there in the tuberculous masses. These are largest in the peribronchial and periarterial regions. In these foci mitotic figures are readily found. Occasional mitoses are found in large mononuclear cells in the peripheral zones of the tubercles. Clusters of polymorphonuclear cells both amphophils and eosinophils are recognizable but are always fragmented and irregular in staining reaction.

The blood vessels and bronchial vessels contain much cellular debris.

Wherever in the section tubercle formation is definite or where groups of cells have undergone hyaline transformation tubercle bacilli are abundant. In the definitely necrotic centers of the tubercles they are present in enormous numbers. In the remaining, more normal interalveolar septa they are scanty for the most part or absent. They are also difficult to find in bronchial and blood vessels even where the contents of these are obviously necrotic cellular remnants.

Rabbit 29.—Bovine bacilli injected. 27 days. Microscopically, discrete and conglomerate tubercles dominate the picture occupying approximately four-fifths of the area of the section. Most of the recognizable cells in the tubercles are large mononuclears. The nuclei stain faintly and the cytoplasm staining also faintly has a hyaline appearance. Many of the tubercles show complete necrosis at the centers. Only in the peripheral zones of some of the tubercles are there small areas where the mononuclear cells stain normally. Even in these areas mitotic figures are not found.

The interalveolar septa adjacent to the tubercles are thickened by mononuclear cell increase. In the small areas which are distinctly removed from tubercles the septa are quite normal in appearance. The alveoli contain very little exudate of any kind.

The lymphoid tissue of the peribronchial regions is almost obliterated by the formation of discrete and conglomerate tubercles. Such remains as are seen show no mitotic figures or other evidence of activity.

The bronchial vessels contain a small amount of cellular debris.

Throughout the section, usually in the tissue between contiguous tubercles, there are found small collections of polymorphonuclear leucocytes. These are almost always in some stage of degeneration.

Tubercle bacilli are readily found within the tubercles and wherever these are necrotic, bacilli are found in large numbers. Outside tubercles bacilli are found with difficulty.

Comparative Description.

A comparison of the foregoing protocols, as well as those not printed, reveals a close similarity in the effects of the two types of bacilli up to the 8th day. The lesions during this period are primarily an increasing interstitial exudation of mononuclear and polymorphonuclear leuco-

cytes affecting the walls of the alveolar spaces. While more or less irregular, the distribution of this lesion may properly be said to be general and diffuse. It is remarkable that the rather extensive changes as revealed by the microscope have caused no definite macroscopic change. The alveoli are very free from an exudation either serous or cellular. Considering the extent or the quality of this interstitial inflammation by itself, and it is the dominant feature of the picture, there is no difference to be made out between the human and bovine series.

In the human series the interstitial inflammation persists with little change until about the 20th day and then gradually is reabsorbed. By the 30th day only traces of it remain in the midzonal portions of the lung, while in the subpleural regions and about the central main vascular trunks the thickened interstitial tissue, more irregular than before, is still plainly evident. In the bovine series the interstitial diffuse inflammation is apparently persistent, but as the tubercles develop gradually ceases to be the dominant feature in the picture. In the last animals of the series the alveolar walls where adjacent to tubercles are thickened, while those more distant are quite normal. This suggests that a great deal of reabsorption of the exudate has taken place in this series also.

Tubercle formation is evident in both series from the 2nd to the 4th day and is equally marked in every respect till the 8th or 10th day. From this point the two series diverge in an extreme degree. In the human series the tubercles enlarge somewhat up to the 18th day, but do not become more numerous, do not become conglomerate, and show no necrosis. After the 18th day the texture of the tubercles becomes looser, individual cells become somewhat hyaline, and the appearances generally suggest that they may be undergoing a reabsorption. By the 30th day they seem definitely less numerous. Those that remain are in some instances quite large and present ill defined connective tissue formation in the peripheral zone. In the bovine series after the 8th day the tubercles increase progressively in number, they coalesce to form conglomerate masses which undergo a process of fusion, and they become necrotic at the centers. Necrosis is evident at 14 days. These changes continue to develop to the end of the series when three-fourths to four-fifths of the area of the sections is occupied by clearly defined tubercles.

Tubercle bacilli are not abundant in either series up to the 8th day, and the two series show about equal numbers during this period. They are found by locating in the diffuse exudate those more definite clusters of mononuclear cells which seem precedent to the formation of well defined tubercles. Associated with those cells, sometimes within them, sometimes apparently between them, the bacilli are found.

After the 8th day bacilli become very difficult to find in the human series. In the bovine series they become increasingly numerous and very rapidly so. By the 14th day in this series, when tubercle formation is well advanced and necrosis has begun, the bacilli are abundant in the tubercle, and after this time are frequently found there in enormous numbers. Even in this series it is very difficult and usually impossible to demonstrate bacilli in the interalveolar tissue apart from all collections which are definitely segregated in such a way as to suggest tubercle formation.

The lymphoid tissue shows a marked hyperplasia in the bovine series from the 2nd day to about the 20th day. After this the lymphoid tissue becomes increasingly occupied by tuberculous tissue and finally almost disappears as such. Throughout this series frank development of tubercles in the lymphoid tissue is later than in the interalveolar exudate. In the human series the lymphoid tissue undergoes no definite hyperplasia, as estimated by the amount of tissue present, and does not become tuberculous to any considerable extent. Mitotic figures are frequently found in cells of lymphocytic type showing that there is here also an increased lymphoidal activity. Occasional large mononuclear cells associated with a few tubercle bacilli were found in lymphoid tissue early in the human series.

Experiments with Killed Bovine Bacilli.

As a check on the series of observations just described a shorter series was carried out in which 1 mg. of the bovine culture previously used was injected into two series of rabbits; in the one series living bacilli were used; in the second series the same suspension was used after heating to 60°C. for 1 hour, a procedure known to be lethal to the bacillus. Animals in each series were killed at 4 day intervals and examined in the same manner as that described.

The series injected with living bacilli developed in all respects in a comparable manner to that of the previous bovine series.

In the series injected with the killed bacilli the diffuse interstitial exudation developed with much the same intensity as that described for the living cultures either bovine or human.   This exudate persisted in a way comparable to that previously observed in the human series, being still well defined at the 20th day which was as far as the series was carried.   Well defined tubercles were not formed, but in the earlier members of the series small clusters of large mononuclear cells were segregated and with these were sometimes found a few tubercle bacilli. Polymorphonuclear cells were much less abundant in the exudate with killed bacilli than in the case of the living, either human or bovine.   They did occur as collections of eosinophils chiefly and for the most part localized in foci of considerable mass in the perivascular tissues, particularly in the peribronchial submucosa.   The lymphoid tissue shows an indefinite hyperplasia in this series marked by an increase in mitotic figures in the small lymphocytes within the nodules, but with no definite increase in the size and number of the nodules.

DISCUSSION AND SUMMARY.

From the foregoing description it is evident that when rabbits are inoculated intravenously with equal amounts of tubercle bacilli of bovine and human type respectively, they are subject to an immediate reaction in the form of an interstitial pulmonary exudation, which, being of equal severity and character does not serve to distinguish the type.

There is an hyperplasia of the lymphoid tissue which is much more pronounced in the bovine series and which may distinguish this type. Opinion on this point may well be reserved until other typical cultures are examined for this response.

The two types are sharply distinguished by the behavior of the tubercle bacillus and by the progression of tubercle formation.   Tubercles are formed by both types and for about 1 week after inoculation they are not distinguishable.   Progressively thereafter those formed as a response to the bovine bacillus become more numerous; they caseate and become conglomerate, finally coming to occupy the major part of the pulmonary tissue and its associated lymph nodes.

With the human type the tubercles do not progress to caseation, do not become more numerous after their first well defined formation, and finally tend to disappear.   The human type bacillus does not multiply considerably, if at all, and disappears early.   The bovine bacillus suffers little or no restraint in growth and finally multiplies enormously. It seems clear that so far as histologic evidence goes the fundamental difference in the reaction of the rabbit to the two types of tubercle bacilli is referable to the ability of the animal to restrain the growth of the human type or to the prevalence of conditions which permit a most vigorous multiplication of the bovine type.   The initial cellular responses seem to be qualitatively of the same order and their quantitative distinctions are for the most part developed coincidently with the manifest growth of the bovine type bacillus.

If we undertake to state the observed results in the terminology of immunity we can say only that the histologic picture discloses a difference in the rate of bacillary multiplication which suggests that a difference in the physiologic requirements for growth of the two types of bacilli is satisfied or unsatisfied, in the respective cases, by the rabbit as host; or on the other hand, that there is a positive growth-restraining action exerted with efficiency against bacilli of the human type.   It is evident that the present observations furnish no points of discrimination between these alternatives.   There is, however, an occasional result of the injection of human type bacilli into rabbits (not seen in this series) which offers a suggestion.

When animals so injected are allowed to live for 2 or 3 months, the lungs at autopsy not infrequently present a few nodules of large size, often 1 cm. in diameter, which are found to be well encapsulated, soft, caseous masses.   These often contain large numbers of tubercle bacilli.   Since we know nothing of the particular conditions which give rise to these rather exceptional formations it is impossible to draw general conclusions from them, but they do suggest that the rabbit is not lacking in the food materials required by the human type bacillus; and that if the more usual suppression of this type is due to failure of its essential nutritives, it is rather a question of the distribution within the animal than an absence which is responsible.   The usual result would then appear to be due to a positive growth-restraining action exerted against the human type bacillus.

Certain other points of interest in the histologic picture described are worthy of comment.

The lymphocytes do not appear as active cells in any preponderant way in either series and they are much less in evidence in the immune case (human type) than in the non-immune (bovine type). This might suggest that the activity of this cell type is a response to infection rather than that it furnished an effective preexisting barrier against infection in this particular case. If the lymphocytes were the most important agents in the immune reaction, it might be expected that they would show an immediate sharp response in the human series.

The large mononuclear type of cell is clearly most closely related physically to the tubercle bacillus within the body of the rabbit and this without distinction as to bacillary type. Foci of these cells are the loci of the disappearing bacilli of human type, and in either the active or necrotic state similar cell collections are the site of the most vigorous multiplication of the bovine bacilli. These cells undoubtedly stand in a central position in any picture which can be drawn of experimental tuberculosis in the rabbit and deserve as a consequence all of the very considerable attention they have received at the hands of numerous observers in recent years.

It has been quite usual of late to consider that the whole of the essential reaction of the animal against tubercle bacilli is carried by the cells of the mononuclear series, either lymphocytes or large mononuclears according to the predilections of the observer. We cannot, however, entirely overlook the presence in very large numbers of polymorphonuclear leucocytes, both amphophilic and eosinophilic, in this experimental series. They are much less prominent in the animals injected with the killed culture and hence can hardly be neglected on the assumption that they are merely a part of a reaction to an indifferent foreign body They are in large measure a reaction to the living organism: whether a primary and direct or a secondary, indirect consequence of its presence we are unable to decide.

These cells are not massed in any regular relationship to the well formed tubercles or to the clusters of mononuclear cells initiating tubercle formation. They are also very much less abundant in the very severe late lesions of the bovine type where enormous numbers of

bacilli are enclosed in the tubercles. It seems possible that the polymorphonuclears are a response to the living free tubercle bacilli as contrasted with either the dead bacilli or the living bacilli segregated in mononuclear cell clusters or in tubercles. They would appear also to be related to something apart from the bacillus itself, either a diffusion or disintegration product, since phagocytosis of bacilli, or the presence of bacilli in close physical relation to polymorphonuclear leucocytes, is so infrequent in general as not to have been observed in this series of experiments.

<div align="center">CONCLUSIONS.</div>

The natural resistance of the rabbit to infection with the tubercle bacillus of human type is apparently referable to a failure of this type of bacillus to multiply in the body of this species to any considerable extent. Exceptionally there are localized lesions (kidneys and lung nodules) which are associated with abundant growth and which show that this failure to multiply may be due to some positive growth-restraining factor rather than to a failure of suitable nutritive materials. There is no qualitative difference in the cellular response to the bacillus of human type contrasted with that to the bovine type, which seems adequate to account for the difference in susceptibility. The eventually more abundant tubercle formation and the degenerative changes occurring in the tubercles formed with the bovine type bacillus seem to be a consequence of the abundant growth of this type; the initial response to equal quantities of the two when injected intravenously being approximately the same both quantitatively and qualitatively.

EFFECT OF AGE ON SERUM LIPOIDS AND PROTEINS.

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Blood serum is endowed with the property of inhibiting the multiplication of fibroblasts and epithelial cells, as has been shown by the experiments of Carrel and Ebeling.[1] It was also found that this growth-restraining power markedly increases with the age of the animal from which the serum is taken.[2] This phenomenon is due chiefly to the lipoids of the serum and also in some measure to the proteins.[3] However, serum also contains certain principles which precipitate with the euglobulin and possess a slight activating effect on the growth of connective tissue cells.[4] In old age, this property of the serum euglobulin was found to decrease.[5] The growth-restraining power of serum in the senescent period is probably due to some change in its lipoids or its proteins, or in both. The experiments reported in this paper were designed to test this supposition, and to determine the nature of the modifications that occur in the protein and lipoid constituents of serum in the course of life.

EXPERIMENTAL.

Sera were obtained from 6 month old chickens and from 4 to 5 year old hens of the same breed. The lipoid and protein fractions were separated and tested for retarding action on the growth of fibroblasts, the lipoid of the serum of the young animal being compared with

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1922, xxxv, 647; xxxvi, 399; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, xlii, 143; *Compt. rend. Soc. biol.*, 1925, xciii, 79.

[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 419; *Compt. rend. Soc. biol.*, 1924, xc, 172. Carrel, A., *Compt. rend. Soc. biol.*, 1924, xc, 1005.

that of the old hen, and the serum protein of the young chicken with that of the old one. The sera were also analyzed for the concentration of various proteins and lipoids.

Method of Preparing the Lipoid and Protein Fractions.

The sera were poured drop by drop, with constant stirring, into three times their volume of 95 per cent alcohol previously cooled to −10°C., and allowed to stand for 2 hours in a freezing mixture. The precipitated protein was centrifuged and washed twice with 95 per cent alcohol, three times with absolute alcohol, twice with a mixture of absolute alcohol and absolute ether, and finally three times with absolute ether. The alcohol and ether had been previously cooled to −5°C. or below, and the protein was separated from them by centrifuging in a tube surrounded by freezing mixture in a brine-cooled centrifuge, so that the temperature did not rise above zero degree throughout the process. The protein fractions of the sera were immediately dried *in vacuo* over sulfuric acid and redissolved in Tyrode solution. As only a very small amount of the protein became insoluble, solutions could usually be obtained equal in protein concentration to that of the original sera. Nitrogen determinations were made and the protein concentrations of the solutions adjusted to the same ratio as that of the original sera.

The alcohol in which the protein was precipitated and the alcohol and ether washings were mixed, filtered, and evaporated to dryness *in vacuo*. Shortly before the preparation of the cultures, the lipoid residue was emulsified in a volume of water equal to the original volume of serum, the pH adjusted, the cryoscopic point determined, and corrections for salt concentration were made.

Method of Testing the Growth-Inhibiting Action of the Protein and Lipoid Fractions.

The above preparations were tested on fibroblasts from a 14 year old strain, the two halves of the same piece of tissue being cultivated, one in the serum lipoid of the old animal and the other in the serum lipoid of the young animal. Since the two halves of the same culture of an old strain of fibroblasts grow at a uniform rate when the media are uniform,[6] any variation in growth would be due to differences in the media. The original sera were compared with each other in the same way, and also the protein fractions from the sera of the old and young animals. The technique described by Ebeling[6] for measuring the relative growth of the tissues was used. The original area and the area after 48 hours incubation were measured, and the rate of growth was calculated by dividing the latter by the former. The culture media consisted of 1 part of plasma, 1 part of either lipoid emulsion, protein solution, or serum, and 1 part of lipoid emulsion, protein solution, or serum containing 5 per cent embryo juice.

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[6] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

Effect of Protein and Lipoid Fractions on the Growth of Fibroblasts.

In every case (Table I), the serum lipoids of the 4 year old hens were more inhibiting to growth than those of the 6 month old chickens. The same is true of the serum proteins (Table II) and also the total

TABLE I.

Comparative Rate of Growth of Fibroblasts in Serum Lipoid from Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in lipoid emulsion		Ratio of growth: $\frac{\text{Young}}{\text{Old}}$	Average ratio
			Young	Old	Young	Old		
1	2414 A	X-144	1431	1430	6.30	5.80	1.10	
					7.20	5.50	1.30	1.25
					3.80	2.80	1.35	
2	2781 A	X-232	2230	2229	3.48	2.64	1.30	
					5.40	2.90	1.85	1.65
					3.85	2.15	1.80	
3	2799 A	X-236	2402	2401	3.45	3.00	1.15	
					3.08	2.47	1.25	1.22
					2.90	2.30	1.25	
4	2823 A	X-241	2431	2432	4.00	3.20	1.25	
					4.10	3.20	1.30	1.28
5	2841 A	X-246	2494	2495	8.30	5.70	1.45	
					5.60	4.10	1.35	1.33
					8.40	7.00	1.20	
6	2857 A	X-249	2528	2529	5.90	4.70	1.25	
					7.20	4.35	1.65	1.53
					10.20	6.01	1.70	
Average..								1.38

sera (Table III). Hence it is evident that changes take place with age in both the lipoid and protein fractions of the serum of an animal, which cause its increased inhibiting action on the growth of fibroblasts. The average ratio of the growth in the serum lipoid of the young animal to that in the serum lipoid of the old animal was 1.38, while the

ratio of the growth in the young serum to that in the old was 1.56. The ratio for the protein fractions was 1.50. While there is a wide variation in the figures from which these average ratios were obtained, due to individual differences in the animals and to the inevitable ex-

TABLE II.

Comparative Rate of Growth of Fibroblasts in Serum Protein from Old and Young Chickens.

Group No.	Culture No.	Chemical Prepara-tion No.	Serum No.		Rate of growth in serum protein		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	2413 A	X-145	1431	1430	7.60	4.40	1.70	
					6.60	5.10	1.30	1.43
					10.00	7.60	1.30	
2	2774 A	X-233	2230	2229	6.65	5.00	1.30	
					8.60	6.90	1.25	1.25
					7.00	5.70	1.20	
3	2800 A	X-237	2402	2401	4.90	3.00	1.60	
					7.00	3.40	2.10	1.83
					6.70	3.70	1.80	
4	2822 A	X-243	2431	2432	4.50	2.60	1.70	
					4.00	3.20	1.25	1.45
					5.40	3.90	1.40	
5	2851 A	X-247	2494	2495	4.25	3.10	1.35	
					6.50	2.70	2.40	1.85
					8.27	4.65	1.80	
6	2863 A	X-250	2528	2529	4.20	3.25	1.30	
					6.00	4.90	1.20	1.20
					6.20	5.50	1.10	
Average..........								1.50

perimental error, the average ratio for the lipoid differs from that of the sera and protein fractions in a manner to be logically expected. It is known[5] that the sera of the young animals contain in addition to inhibiting substances a small amount of growth-stimulating substance precipitated with the euglobulin by CO_2. This stimulating substance

is absent from the sera of the older animals.[5] Thus, in Ebeling's experiments, the difference in rate of growth of fibroblasts in the CO_2 precipitate of sera of old and young chickens was 0.18.[5] The ratio 1.56 for the sera is due, then, to two factors, the smaller quantity of inhibiting substance in the sera of the younger animals, and the pres-

TABLE III.

Comparative Rate of Growth of Fibroblasts in Serum of Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in serum		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	1448 C	X-142	1431	1430	8.40	7.60	1.10	
					6.95	5.10	1.35	1.28
					9.30	6.50	1.40	
2	2773 A	X-231	2230	2229	7.10	3.50	2.00	
					5.70	3.35	1.70	1.80
					6.00	3.55	1.70	
3	2813 A	X-235	2402	2401	4.50	2.88	1.55	
					8.90	4.25	2.10	1.62
					7.95	6.70	1.20	
4	2802 A	X-242	2431	2432	5.30	3.90	1.35	
					6.00	3.80	1.60	1.40
					8.60	6.90	1.25	
5	2842 A	X-245	2494	2495	13.90	7.20	1.90	
					9.40	6.30	1.50	1.65
					7.90	5.10	1.55	
6	2873 A	X-252	2528	2529	4.80	3.54	1.35	
					9.75	4.58	2.10	1.63
					5.40	3.74	1.45	
Average..								1.56

ence of a stimulating substance. Since the latter would be absent in the lipoid fractions, a lower ratio for these would be expected and was actually found.

The data given here do not show how much greater is the inhibiting action of the lipoid than that of the protein fraction, since the lipoid

and the protein were not tested on the two halves of the same tissue, and the growth of two different pieces of tissue cannot be compared, because of the marked effect of their previous culture medium and their inherent growth activity. The relative effect of the lipoid and protein was demonstrated in previous experiments,[3] in which the lipoids proved even a little more inhibiting than the sera from which they were derived, while the protein fractions were much less inhibiting than the original sera. It is interesting to note that although the protein is less inhibiting than the lipoid, the rates at which their inhibiting action increases with age seem approximately equal.

TABLE IV.

Concentration of Protein in Sera of 6 Month and 4 to 5 Year Old Chickens.

6 mos. old			4 to 5 yrs. old		
Serum No.	Per cent nitrogen	Per cent protein	Serum No.	Per cent nitrogen	Per cent protein
1431	0.455	2.85	1430	0.885	5.53
2230	0.462	2.89	2229	0.690	4.32
2402	0.621	3.88	2401	0.912	5.70
2431	0.634	3.96	2432	0.707	4.42
2494	0.670	4.18	2495	0.547	3.42
2528	0.532	3.33	2529	0.709	4.44
Average...	0.562	3.52		0.742	4.64

Concentration of Protein and Various Protein Fractions in Sera of Old and Young Animals.

The sera used for the growth experiments were tested in the course of the work for their concentration of protein (Table IV). Here again, the individual variations are large, but it is evident that in general the sera from the older animals contain a higher concentration of protein, the average being 0.742 per cent nitrogen for the 4 to 5 year old hens, and 0.562 per cent nitrogen for the 6 month old chickens. The ratio of the average concentrations was 1.32. This corresponds fairly closely to the ratio that represents the inhibiting action on growth, *i.e.*, the rate of growth in the protein fractions is approximately inversely proportional to the protein concentration. Since serum contains a growth-stimulating substance which precipi-

tates with CO_2, a determination of the concentration of protein not precipitated by CO_2 was made on sera from old and young chickens. Here again, the concentration was found to be greater in the sera from the older animals (Table V). Hence, it is evident that the

TABLE V.

Concentration of Various Protein Fractions of the Sera of Old and Young Chickens.

Serum No.	Per cent total protein	Per cent protein not precipitated by CO_2	Per cent protein precipitated by CO_2. By difference	Per cent globulin in CO_2 filtrate	Per cent albumin
3 to 5 mos. old					
3007	3.13	2.92	0.21	1.45	1.47
3020	3.22	2.50	0.72	1.21	1.29
3029	2.29	1.87	0.42	0.56	1.31
3036	3.04	2.53	0.51	0.94	1.59
3040	2.52	2.06	0.46	0.90	1.16
3046	3.56	2.26	1.30	1.13	1.13
3053	3.28	2.76	0.52	1.42	1.34
3064	3.31	2.66	0.65	1.35	1.31
Average............	3.04	2.45	0.60	1.12	1.33
4 to 5 yrs. old					
3006	4.89	3.19	1.70	1.58	1.61
3021	2.99	2.41	0.58	1.00	1.41
3030	4.89	3.69	1.20	2.37	1.32
3035	4.12	3.15	0.97	1.31	1.84
3039	4.55	2.20	2.35	1.17	1.03
3045	3.63	2.53	1.10	1.25	1.28
3052	3.94	3.14	0.80	1.45	1.69
Average............	4.14	2.90	1.24	1.45	1.45
Ratio: $\dfrac{\text{Old}}{\text{Young}}$	1.36	1.18	2.06	1.29	1.09

increase in the inhibiting action of the protein with age is due not only to the disappearance of the small quantity of stimulating substance in the CO_2 precipitate, but also to a change in the concentration of inhibiting protein.

Determinations were also made of the concentration of albumin.

and of pseudoglobulin[7] remaining in the filtrates from the CO_2 precipitation. Both of these proteins were found to occur in greater concentration in the sera of the old animals (Table V). Therefore, it is not possible to attribute the increased inhibiting action of the protein to either one of these proteins by itself. It is more probably due to their joint action. Even the quantity of euglobulin precipitated by CO_2 is greater in the sera of the older animals (Table V), indicating the probability that the euglobulin itself is not the stimulating substance, but rather some substance carried down with it. No rigid conclusion should be drawn here, however, since it is possible that this precipitate might contain some of the fibrin of the plasma from which the serum was made.

There is considerable variation among the sera of the individual animals of the same age in the concentration of the different protein fractions. Duplicate determinations on any one serum agreed closely, however, showing that this phenomenon is due to actual differences in the animals, and not to the experimental technique. The same variation is noted in the growth of fibroblasts in the sera of animals of the same age. Therefore, although the average results are obtained from figures varying to quite an extent, they indicate without any doubt greater inhibiting action in the serum protein of the older animals, and also a higher concentration of protein in the sera as the age of the animals increases. It seems probable that this inhibiting action is not due to any one protein, but rather to the protein as a whole.

Concentration of the Total Lipoid, Lecithin, and Cholesterol in Sera of Old and Young Animals.

An examination of the changes taking place in the lipoid of the serum with increasing age is even more important than that of the protein, since the lipoid is chiefly responsible for the inhibiting action. The total lipoid was determined in the sera of chickens 6 months old and 4 to 5 years old, the lecithin for 3 month, 6 month, and 4 to 5 year old chickens, and cholesterol for 3 month and 4 to 5 year old chickens. The total lipoid was determined as follows:

5 cc. of serum were poured drop by drop into 75 cc. of a mixture of alcohol and ether (3:1). The solution was heated to boiling, cooled, made up to 100 cc ,

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[7] This fractionation was made by the use of sodium sulfate.

and filtered. An aliquot portion of the filtrate (75 cc.) was taken for analysis. Three times its volume of absolute ether was added. The precipitate obtained had no inhibiting action on fibroblasts, while the lipoids remaining in solution showed the usual retarding action. After centrifugation, a large aliquot (250 cc.) was evaporated to dryness in a tared flask, and the residue weighed. To ascertain whether the residue contained salts that would invalidate the results, the residue was redissolved, transferred to a platinum crucible, and ashed. A negligible amount of ash remained (0.0014 gm.), showing that the salts had been satisfactorily removed. The lipoid itself would, of course, yield a small quantity of ash. Therefore, no error from this source needs to be considered. In developing the above procedure, it seemed desirable to use a method resembling as nearly as possible that employed in preparing the lipoid fraction for the culture medium, especially since the usual methods of determining total lipoid are not very precise. Sufficiently large quantities of serum were used (5 cc.) to diminish the experimental error to less than 1 per cent.

TABLE VI.

Concentration of Total Lipoid in Sera of 6 Month Old and 4 to 5 Year Old Chickens.

6 mos. old		4 to 5 yrs. old	
Serum No.	Weight per 100 cc. gm.	Serum No.	Weight per 100 cc. gm.
2755	0.800	2754	1.133
2774	0.815	2773	1.055
333C	0.992	332C	0.993
2835	0.895	2834	1.042
		2848	1.072
Average..........	0.876		1.059

Lecithin in the sera was determined at first by the method of Randles and Knudson,[8] and later by Whitehorn's[9] modification of that method. The Whitehorn modification gave more uniform results than the technique of Randles and Knudson on duplicate determinations. The method of Bloor[10] was found to be the most suitable for determining cholesterol. As other experimenters have found, the colors did not always match the standard. Both a cholesterol standard and the inorganic standard of copper sulfate and sodium dichromate of

[8] Randles, F. S., and Knudson, A., *J. Biol. Chem.*, 1922, liii, 53.

[9] Whitehorn, J. C., *J. Biol. Chem.*, 1924, lxii, 133.

[10] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

McMaster[11] were used. While these figures involve quite a large experimental error, there was no difficulty in distinguishing between

TABLE VII.

Concentration of Lecithin Phosphorus in Sera of Chickens of Different Ages.

3 mos. old			6 mos. old			4 to 5 yrs. old		
Serum No.	Mg. phosphorus per 100 cc. serum	Average	Serum No.	Mg phosphorus per 100 cc. serum	Average	Serum No.	Mg. phosphorus per 100 cc. serum	Average
2871	4.80 5.40	5.10	2817	5.90	5.90	2754	6.70 6.50	6.60
2870	5.90 4.95	5.43	2333	5.00 5.10	5.05	2773	7.20 7.50	7.35
2859	4.50 4.60	4.55	2774	6.40 6.30	6.35	332C	6.90 7.50	7.20
2849	3.45 4.85	4.15	2755	6.10 5.40 5.20	5.57	2818	6.70 6.70	6.70
2835	4.80 4.60	4.70				2834	7.80 7.50	7.65
						2848	7.50 7.60	7.55
						2857	9.30	9.30
						2869	7.30 6.70	7.00
						2868	7.40 7.30	7.35
Average.........		4.78			5.72			7.41

the quantity of cholesterol present in the sera of the old and young chickens.

The results (Tables VI to VIII) show that there is an increase in

[11] McMaster, P. D., *J. Exp. Med.*, 1924, xl, 25.

TABLE VIII.

Concentration of Cholesterol in Sera from 3 Month and 4 to 5 Year Old Chickens.

3 mos. old			4 to 5 yrs. old		
Serum No.	Mg. cholesterol per 100 cc. serum	Average	Serum No.	Mg. cholesterol per 100 cc. serum	Average
2870	282 226 338	282	2869	166 188 200	185
2871	200 165 100	155	2868	83.0 95.3 95.3	91
2891	200 198	199	2889	75 68	71
2892	273 250	261	2904	187 220 250	219
2892	273 238	255	2905	107 107 99	104
2859	157 144 144	148	2857	170 200 200	190
2907	340 340 322	334	2754	192 205 196	198
2906	191 178 166	178	2818	161 134 128	141
2870	215 201 213	210	2834	87 80 86	84
Average................		225			143

the total lipoid and also in the quantity of lecithin as the age of the chicken increases, but a decrease in the cholesterol.[12]

The total lipoid in the sera of the 4 to 5 year old chickens was 1.06 per cent and in the 6 month old, 0.876 per cent, giving a ratio of 1.21. The concentration of phosphorus due to lecithin was 4.78 mg. per 100 cc. in the sera of the 3 month old chickens, 5.72 mg. per 100 cc. in the sera of the 6 month old chickens, and 7.41 mg. per 100 cc. in the sera of the 4 to 5 year old chickens. The ratio of the 4 to 5 year old to the 6 month old animals was 1.29. These figures are in general accord with the results on growth in the serum lipoids of animals of these ages, and indicate that the inhibiting action is due, at least partly, to the lecithin which increases in concentration in old age. The fact that the concentration of cholesterol decreases as age increases, 225 mg. per 100 cc. at 3 months to 143 mg. per 100 cc. at 4 to 5 years, shows the necessity of further investigation in this field. The differences with age are so small, in comparison with the individual variations between animals of the same age, that it is impossible as yet to draw any conclusions concerning the part cholesterol plays in the increased inhibiting action of serum lipoids with age. As yet, the separate lecithin and cholesterol fractions of the sera have not been investigated in relation to their action on growing fibroblasts. A study of the action of pure cholesterol is now being undertaken.

<center>DISCUSSION.</center>

It is evident from the foregoing experiments that the changes in serum accompanying an increase in age, which render it more inhibiting to the growth of fibroblasts, occur in both the lipoids and the proteins of the serum. Although the serum protein is less inhibiting than the serum lipoid, the rate at which their retarding actions augment with age is approximately the same and also the same as the rate at which the retarding action of the serum itself augments with age. The change taking place in the protein fraction is due to the disappearance of a small quantity of growth-stimulating substance and also

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[12] Roffo found an increase in both lecithin and cholesterol in rats between the ages of 3 and 5 months, but he made no examination of the sera of older rats. Roffo, A. H., *Compt. rend. Acad.*, 1925, clxxx, 1529.

an increase in the quantity of inhibiting protein. The concentration of each protein fraction of the serum is also greater as age advances. The increased inhibiting action of the serum lipoid in old age is associated with a higher concentration of total lipoid and of lecithin, and a smaller content of cholesterol than is present in the sera of young animals. An investigation of the action on fibroblasts of the lecithin and cholesterol fractions of the lipoid and of pure lecithin and cholesterol is necessary.

It would be interesting to know the mechanism by which these chemical substances act upon the cells and restrain their multiplication. Under normal conditions, the humors of the animal acquire a delicate balance between the growth-stimulating substances and those retarding growth, an understanding of which might give some information concerning abnormal conditions in which this balance is not maintained.

Probably there is also a delicate balance between conditions producing growth and those under which functioning of cells takes place. It is known that heart tissue kept in a medium unsuitable for growth will continue its muscular functioning, but this stops when nutritive substances are added to the tissue and growth takes place. In connection with this, it is interesting to note that the lipoids retard growth of tissues, but that an isolated heart perfused continuously with Ringer solution will cease beating, due to the loss of some substance removed by the Ringer solution, but will have that function restored on the addition of a small amount of the ether extract of serum.[13] Lecithin produces the same effect. As yet these phenomena are far from being understood.

The data at hand are also too meager to venture on any explanation of how lipoid and protein inhibit growth. However, since it has been discovered[14] that fibroblasts proliferate rapidly in proteose solutions and peptic digests of proteins, and that they live and multiply in the protein of embryo juice, it seems probable that this protein is first hydrolyzed to the proteose stage by means of enzymes either in the

[13] Clark, A. J., *J. Physiol.*, 1913–14, xlvii, 66.

[14] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503. Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060. Carrel, A., and Baker, L. E., *Compt. rend. Soc. biol.*, 1926, xcv, 359.

embryo juice or within the cells. It is known that serum exerts an antienzymatic action. It is possible, therefore, that the inhibiting action of serum on growth may be associated with its antienzymatic action, which would retard the production of nutritive hydrolytic products.

SUMMARY.

The increased inhibiting action of serum with age on the growth of fibroblasts has been shown to be due to changes in both the lipoids and the proteins.

Although the serum protein is less inhibiting than the serum lipoid, the rate at which their inhibiting action augments with age is approximately the same.

The change in the serum protein with age is due to the disappearance of a small quantity of growth-stimulating substance, and also to an increase in concentration of inhibiting protein. The concentration of all protein fractions becomes larger as age advances.[15]

The greater inhibiting action of the lipoid is associated with a higher concentration of total lipoid and lecithin, and a smaller content of cholesterol as the animal grows older.

The hypothesis is suggested that the inhibiting action of the serum is associated with its antienzymatic action.

[15] It is well known that the protein of serum increases as age advances. Investigators differ in their findings on the relations between age and the different protein fractions. Wells, C. E., *J. Biol. Chem.*, 1913, xv, 37. Hatai, S., *J. Biol. Chem.*, 1918, xxxv, 527. Toyama, I., *J. Biol. Chem.*, 1919, xxxviii, 161.

THE BACTERICIDAL PROPERTY OF COW'S MILK.

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Many have commented on the bactericidal properties of raw milk. It is well known that when certain bacteria are introduced into fresh raw milk their numbers decline for a time and subsequently multiply. Hesse[1] noted that *B. typhosus* and the cholera vibrio were prevented from multiplying in raw milk. Park[2] observed that the number of bacteria decreased in milk kept for 24 hours at 42°F., the same sample stored at 50°F. revealed a moderate increase in bacteria, and when the holding temperature reached that of the room or the incubator the bacterial increase was marked. Heinemann[3] found that milk contains for certain species of organisms a bactericidal substance, but the growth of other organisms is not inhibited. He further pointed out that the substance was destroyed at the boiling point or by heating to 60°C. for 30 minutes.

Rosenau[4] studied the phenomenon in considerable detail. He concluded that there is a diminution of the organisms in fresh raw milk and compared the action to that of a weak disinfectant and showed that the number of bacteria decreased markedly during the first 8 or 10 hours of incubation but rose rapidly thereafter. The action was prolonged but less rapid at 15°C. He attributes the apparent decrease largely to agglutination and calls attention to the possibility of phagocytosis by the leucocytes as an additional explanation for the decline.

More recently Chambers[5] showed that milk contains a definite bactericidal property which is destroyed by heating at 80° or 90°C. for 2 minutes. He found that the action was specific and depended on both the cow and the species of bacteria employed. He failed to find a relationship between agglutination and growth inhibition except that they were both destroyed by heat. It was pointed out that the lactic acid types of organisms were not inhibited in milk.

[1] Hesse, W., *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 238.

[2] Park, W. H., *N. Y. Univ. Bull. Med. Sc.*, 1901, i, 71.

[3] Heinemann, P. G., The kinds of bacteria concerned in the souring of milk, Chicago, 1903.

[4] Rosenau, M. J., and McCoy, G. W., *U. S. Pub. Health and Marine Hosp. Service, Bull. 41*, 1908, 449.

[5] Chambers, W. H., *J. Bact.*, 1920, v, 527.

Sherman and Curran[6] inoculated fresh milk with young cultures of *Streptococcus lacticus*. There was lag in the fresh milk cultures for 30 minutes, but in the control tubes in which sterile milk was employed no lag occurred.

Hanssen[7] found that fresh raw milk would inhibit the growth of *B. typhosus* and *B. parathyphosus* B for 1 to 4 hours at 37°C. When the inoculated milk was kept at the temperature of the room the decrease was slower but more prolonged. He further showed that milk from single cows varied greatly in the inhibiting substance at different periods. This he attributes to variations in the ration, since he infers that the substance is due to the concentration of oxidizing enzymes in the milk. The enzymes supposedly originate in the food and their concentration in the milk is dependent on their concentration in the ration. He also showed that milk heated to 63°C. for 30 minutes and to 70°C. for 15 minutes still retained its inhibitory activity. 75°C. for 15 minutes inactivated the substance. This fact indicated to him that the substance in the milk was not derived from the alexin of the blood.

The phenomenon of bacterial decrease has been explained in several ways: lack of adaptation of the organisms employed (Stocking[8]), bacterial lag, agglutination-phagocytosis, and finally a definite property of fresh raw milk. A number of workers have failed to observe the phenomenon of growth inhibition in fresh raw milk.

If we should assume that milk possesses a definite bactericidal substance, perhaps its true purpose has been overlooked. Many have commented on its function, especially in regard to the keeping qualities of milk. That such is not its true function seems obvious. It is clear that the udder contains a relatively limited flora of organisms which have adapted themselves to their environment. Among the organisms usually encountered are streptococci and various types of micrococci; both are frequently associated with mastitis. It is also probable that many bacteria capable of multiplying in milk, such as the intestinal or vaginal flora, frequently come in contact with the ends of the teats; yet relatively rarely are organisms of this class encountered in the normal udder. When they occur they are usually associated with udder inflammation. Again, we are confronted with the problem of resistance to udder infection. Certain cows remain in the herd for several years without developing udder disease while

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[6] Sherman, J. M , and Curran, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 15.

[7] Hanssen, F. S., *Brit. J. Exp. Path.*, 1924, v, 271.

[8] Stocking, W. A., *Rep. Conn. Agric. Exp. Station*, Storrs, 1904, 89.

others suffer from repeated attacks. As far as known, the blood and milk of the resistant group contain no more agglutinins than the same fluids of the susceptible animals. That the bactericidal substance in milk might be of considerable assistance in preventing the growth of organisms within the udder seemed worthy of investigation, particularly if it would throw light on the problem of resistance to mastitis. With these points in view a number of observations were made.

EXPERIMENTAL.

Methods.—Since our problem concerned itself with the possible influence of the inhibitory substance within the udder, we decided to test the milk against the nonhemolytic mastitis streptococcus, the organism we have found most frequently in udder infection. Our Culture 55 was isolated in 1917 and since that time has been carried on horse blood agar or plain agar slants. It is usually transferred four or five times a year. Presumably it has adapted itself to growth on artificial media but readily multiplies in milk. It has the further advantage of agglutinating slowly even with high titered agglutinin.

Milk was always drawn directly into sterile bottles and all precautions were taken to avoid contamination. Generally the samples were taken from the middle portions of the milking. The milk after chilling was rapidly centrifuged and a portion of the liquid between the cream line and sediment withdrawn. In this way a relatively fat-free milk containing very few cells and a limited number of organisms was obtained. The milk was then distributed in amounts of 1 cc. in sterile agglutination tubes containing a glass bead. A sample of boiled or autoclaved milk was also distributed in the same way for control purposes.

All tubes were then inoculated with one loop of an 18 hour broth culture of the mastitis streptococcus, which was diluted 100 times with broth. Each tube was shaken before incubation and at half-hourly intervals throughout the experiment. The contents of the tubes were withdrawn at various times and plated with 12 cc. of 2 per cent agar prepared from veal infusion. The number of colonies was counted after 24 hours incubation at 38°C.

Our methods largely eliminated the cells present in the milk as well as decreased the number of udder organisms. The repeated shaking of the tubes containing the glass beads prevented agglutination, since clumps could not be detected on microscopic examination.

When blood serum was used it was handled in a similar manner. It was always freshly obtained and relatively free from cells.

Experiment 1.—In the herd to which we have had access, we had the opportunity of testing the bactericidal action of the milk from four groups of cows. The first group consisted of native cows which had been in the herd for several years and had no history of mastitis. The second group had been injected on four occasions with killed cultures of the mastitis streptococcus. They had been recently im-

TABLE I.

The Bactericidal Activity of the Milk and Serum of 8 Cows for the Non-Hemolytic Mastitis Streptococcus.

Cow No.	Group	Milk + culture of mastitis streptococcus Number of colonies				Blood serum + culture of mastitis streptococcus Number of colonies				
		At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	After 20 hrs.
1	Resistant	8,448	6,464	6,784	6,400	8,704	7,040	6,336	4,864	2,688
2	"	8,960	8,320	6,528	6,218	8,448	8,768	6,786	2,592	42
Sterilized milk	Control	9,344	24,768	Innumerable	Innumerable					
3	Vaccinated	4,928	4,540	3,520	20,100	5,120	5,824	4,480	4,350	Innumerable
4	"	4,740	3,710	3,392	22,000	4,992	5,760	5,120	2,560	41,600
Sterilized milk	Control	5,370	9,792	Innumerable	Innumerable					
5	Young cows in first lactation period	4,480	3,520	3,584	28,800	4,480	3,456	4,160	3,648	Innumerable
6	"	4,480	3,390	4,800	Innumerable	4,160	3,748	3,712	4,544	"
Sterilized milk	Control	4,860	9,090	Innumerable	"					
7	Cows susceptible to mastitis	4,416	3,588	3,584	"	4,416	4,864	4,544	5,376	"
8	"	4,544	3,904	3,392	"	4,288	4,991	3,776	2,240	"
Sterilized milk	Control	4,352	10,760	Innumerable	"					

ported from Tennessee and their udders were free from non-hemolytic streptococci when introduced into the herd. The third group consisted of young native animals in their first lactation period. The fourth series were animals which had had several attacks of mastitis. The findings were about the same for all the animals in each group so that only the details of two tests are given. The details of the results of the bactericidal activity of both the milk and blood serum are given in Table I. In these observations sterilized milk from the laboratory supply was used for control purposes.
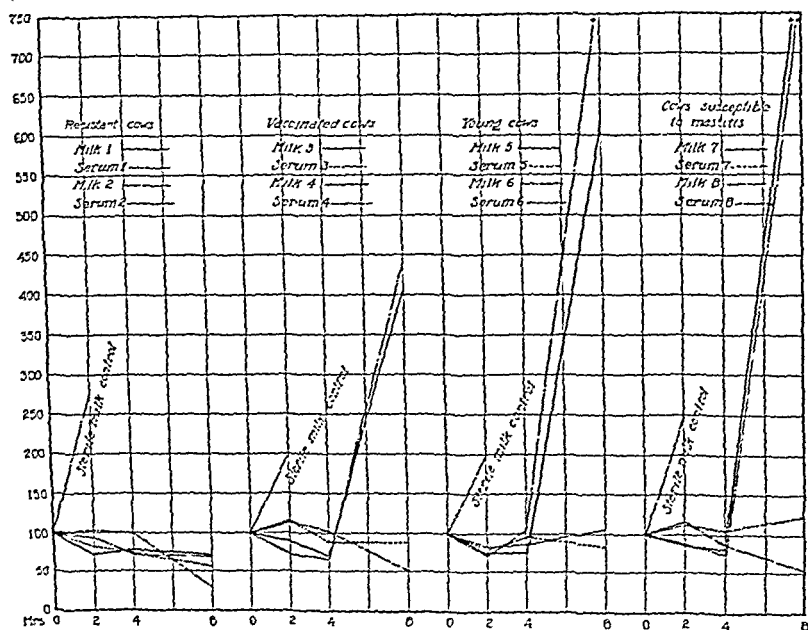
CHART 1. The inhibition of the mastitis streptococcus in milk and blood serum of resistant, vaccinated, an d young cows and of cows susceptible to mastitis.

The data given in Table I have been plotted for the purpose of comparison and are shown in Chart 1. In the charts the number of colonies counted in the samples of both the raw and the boiled milk in each series have been added and an average struck; this represents 100 per cent, and departures from this line are recorded in terms of per cent. The results with the serum are given in the same way.

Both the table and the chart illustrate certain features. The milk

from all groups reacts in a similar manner. There is a sharp decline in the number of organisms during the first 4 hours of incubation, which is common to all groups. After this time there is a rapid multiplication particularly in the instance of the young cows and those susceptible to mastitis. The vaccinated group are about midway between the most resistant animals and the young and susceptible cows. The inhibition of the milk of the resistant cows is marked, since there is no multiplication during the first 8 hours. In sharp contrast is the rapid increase in the number of streptococci in the sterilized milk control. Here the number of organisms nearly doubled during the first 2 hours.

TABLE II.

The Bactericidal Activity of the Milk of a Resistant Cow and of a Young Cow Early in Its First Lactation Period.

			Streptococci present		
	At once	After 2 hrs	After 4 hrs.	After 6 hrs	After 8 hrs.
Milk of resistant Cow 1	5,200	4,392	4,928	4,608	12,608
Boiled milk of Cow 1	5,000	48,900	Innumerable	Innumerable	Innumerable
Milk of young Cow 9, early in first lactation period	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 9	5,068	36,990	Innumerable	Innumerable	Innumerable

It will be noted that the serum was in all cases inhibitory during 8 hours. This in itself is of little significance since the organism has not been trained to grow in serum and hence the lag period is relatively long. However, when the incubation period was increased to 20 hours, it was found that the serum of resistant Cows 1 and 2 was distinctly bactericidal and that of Cow 4 had definite inhibitory properties.

Inasmuch as in these observations both the milk and the blood were obtained in the morning and tested during the day, no opportunity was afforded for observations in the 6th hour, perhaps a critical point. This point has been covered in Experiment 2.

Experiment 2.—The milk of resistant Cow 1 and a young cow early in the first lactation period was obtained and employed in the manner outlined. In this and

ensuing observations the control consisted of a portion of the milk boiled for 5 minutes. The data have been tabulated in Table II.

From Table II it is clear that the inhibitory substance may be about as active in the milk of a young cow early in its first lactation period as that manifested in the secretion of an old cow which has passed through several lactation periods.

The behavior of the streptococcus in boiled milk affords a sharper contrast than the use of the stock milk medium which has been autoclaved. The former apparently represents an ideal medium, since it is the fresh product boiled for 5 minutes. The stock medium is prepared from commercial skim milk from the same dairy; it is older when separated and requires titration. It is of course heated to a higher temperature for a longer period.

Thus far it has been shown that the bactericidal substance is present to a varying degree in the milk of all cows examined. In addition to these reported, the milk from ten others has been examined with practically identical results. There is always a period from 4 to 8 hours in which the streptococcus fails to multiply. We are inclined to the opinion that during this period there is actual diminution succeeded by a period of multiplication. The streptococcus has thus far failed to coagulate the raw milk before the 8th hour, and in samples possessing strong inhibitory activity there is no coagulation at the end of 24 hours. From a number of observations we are convinced that most of the inhibitory substance is utilized during the first 8 hours, since as soon as the streptococcus begins to increase it continues to do so at a rapid rate. This is well borne out in Table I. Both the boiled milk and the sterilized milk controls are always definitely thickened at 8 hours and often firmly coagulated.

From Table I it appears as if there was a parallel between the activity of the blood serum and that of the milk; it seemed likely that the substance originated in the blood and appeared in a diluted form in the udder. One or two points, however, indicate that this explanation is not entirely true. If the substance originated in the blood, it might be possible to increase its concentration in the milk by immunization. The injection of dead cultures of streptococci into the cows of Group 2 (Table I) did not increase the concentration in the udder

above the usual level.   The milk of young cows early in their first
period of lactation compared in its inhibitory effect with that from the

TABLE III.

The Inhibitory Effect of Milk on Culture 55 and on a Freshly Isolated Strain of Mastitis Streptococci.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk of resistant Cow 1 +Culture 55	6,336	4,800	5,120	6,720	9,185
Boiled milk of Cow 1 + Culture 55	7,260	20,160	Innumerable	Innumerable	Innumerable
Milk of Cow 1 + freshly isolated streptococci	7,168	5,788	6,912	5,760	41,520
Boiled milk of Cow 1 + freshly isolated streptococci	7,296	16,588	Innumerable	Innumerable	Innumerable
Milk of young Cow 10, early in first lactation period + Culture 55	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 10 +Culture 55	5,068	36,992	Innumerable	Innumerable	Innumerable
Raw milk of Cow 10 + freshly isolated streptococci	1,344	1,241	1,088	11,980	19,120
Boiled milk of Cow 10 + freshly isolated streptococci	1,197	30,538	Innumerable	Innumerable	Innumerable

others.   It is to be presumed that these cows had been exposed to
infection for too short a period to acquire resistance to the mastitis
streptococcus.

Since we were convinced that the raw milk of all the cows contained a definite substance which inhibited the growth of the streptococcus, we decided to continue the study with the hope of throwing some light on its properties and its probable source. In Experiment 3 we attempted to show the influence of the culture on the inhibitory substance. The inhibition against Culture 55, previously referred to, and a freshly isolated strain of the mastitis streptococcus in the second culture generation were compared. The results are recorded in Table III.

It is apparent that the inhibitory substance in the milk of Cow 1 is a little more effective against the culture which has been carried on artificial media for a longer period than against the freshly isolated strain, although in the latter instance there is well marked inhibition. In the case of Cow 10, a young animal early in the first lactation period, the effect of the addition of the freshly isolated streptococcus is more pronounced, for inhibition occurred only within the first 4 hours. The experiment also indicates that the freshly isolated strains are better adapted for growth in the raw milk than the older strain isolated several years ago and since cultivated on artificial media.

Although the growth-inhibiting effect of whole or skim milk has been commented on by many, we have failed to find any reference whether the agent is contained in the whey. To test the effect of whey a number of experiments were made. Experiment 4 affords an example.

Experiment 4.—Commercial rennet tablets were ground and dissolved in sterile salt solution in the proportion of 2 tablets to 20 cc. of salt solution. The solution was sterilized by filtration through a Berkefeld candle N. 2.5 cc. of the filtrate was added to 50 cc. of fresh milk largely freed from fat, and after mixing was placed in a water bath at 38°C. Within 10 or 15 minutes sufficient whey was obtained for the tests. As a control procedure 2.5 cc. of salt solution was added to 50 cc. of the milk and the mixture heated in the water bath at 38°C. A third portion of the milk was boiled for 5 minutes. The whey, raw milk, and boiled milk were distributed in the usual manner and inoculated with the culture. The inhibitory effect of the whey and of the milk is given in Table IV.

It is apparent that whey contains the inhibitory substance to about the same extent as the milk. It cannot be said that the substance is greatly concentrated by the removal of the casein.

It has been shown by one of us[9] that milk will react to a relatively uniform level with a specific cow serum precipitin. The milk of Cow 1 used in this experiment reacted with cow serum precipitin at a maximum dilution of 1:640. The whey reacted at a dilution of 1:2,560. Therefore, in the process of rennet coagulation there is a considerable concentration of blood protein in whey. If the inhibitory agent comes from the blood then it is to be expected that it would be considerably concentrated within the whey. The protocols submitted in Table IV show that this is not the case.

Chambers demonstrated that 80° or 90°C. for 2 minutes would destroy the bactericidal effect of milk. Heinemann stated that

TABLE IV.

A Comparison of the Inhibitory Effect of Raw Milk and of Whey on the Mastitis Streptococcus.

	Streptococci present					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs	After 20 hrs.
Milk of Cow 1	5,200	4,392	4,928	4,608	12,608	Innumerable
Whey of Cow 1	5,440	4,224	4,608	4,032	8,896	"
Milk of Cow 11	5,440	4,160	5,632	5,760	28,800	"
Whey of Cow 11	5,696	5,440	4,352	8,064	28,224	"
Combined milk of both cows, boiled for 5 min.	5,000	48,960	Innumerable	Innumerable	Innumerable	"

60°C. for 30 minutes would destroy the substance. Rosenau claimed that the inhibitory effect for *B. lactis aerogenes* was weakened at 55°C. and almost destroyed at 60°C. On the other hand, 60°C. did not affect the inhibition against typhoid. Hanssen states that the inhibitory substance for *B. typhosus* resists a temperature of 63°C. for 30 minutes and 70°C. for 15 minutes, although it is inactivated at 75°C. for 15 minutes. Since there is some difference of opinion in regard to the temperature required to inactivate or destroy the substance in milk, it was desirable to try the effect of various temperatures. For purposes of comparison we also observed the effect of

[9] Jones, F. S., *J. Exp. Med.*, 1926, xliii, 451.

the same temperatures on a cow blood serum of considerable bactericidal activity. It also seemed to us that if it was possible to show that the substance in milk was more or less resistant to heat than that of the blood, it would be regarded as additional evidence that they were different substances. That this really proved to be the case is brought out in Experiment 5.

Experiment 5.—Milk from Cow 13 which would inhibit growth of the mastitis streptococcus during the first 8 hours of incubation was obtained as usual. It was distributed in tubes in amounts of 10 cc. The contents of one tube was used in its raw state, another tube was boiled for 5 minutes, and the others were heated for 20 minutes at temperatures of 56°, 58°, 60°, 62°, 65°, 70°, and 80°C. The milk was then cooled and distributed in amounts of 1 cc. in the small tubes containing glass beads and inoculated with the culture. Cow 1 was bled in the afternoon and the blood stored in the refrigerator overnight. The serum was clear and free from cells. It was distributed in amounts of 7 cc. One tube was not heated. The others were heated for 20 minutes in the water bath at temperatures of 56°, 58°, 60°, 62°, and 65°C., and then all were inoculated with the culture. In this experiment we used a strain of the streptococcus which had been rapidly passed through broth which contained 50 per cent cow serum heated at 60°C. for 30 minutes. The culture was of the same age as the one used to inoculate the milk. The data are given in Table V.

It is apparent that the bactericidal factor for the mastitis streptococcus in the blood serum is inactivated at 56°C. With the increase in temperature inhibition of the substance is more marked. The serum heated at temperatures from 56° to 62°C. reveals no gross changes, but at 65°C. it becomes slightly opalescent; above 65°C. it coagulates. In contrast is the higher temperature required to inactivate the inhibitory agent in milk; temperatures ranging from 56° to 62°C. fail to affect it. When the temperature was increased to 65° or 70°C. for 20 minutes there was some impairment, although milk so treated proved inhibitory during the first 6 hours. 80°C. for 20 minutes is as efficient in inactivating the substance as boiling for 5 minutes.

The difference in the temperature required to inactivate the bactericidal substance in blood serum and in milk strongly suggests that the substances are different.

It is well known that antibody and blood proteins pass from the

TABLE V.

The Effect of Various Temperatures on the Bactericidal Activity of Milk and Cow Serum.

	Milk					Blood serum					
	Number of colonies					Number of colonies					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs	After 8 hrs	At once	After 2 hrs	After 4 hrs	After 6 hrs.	After 8 hrs.	After 20 hrs.
Unheated	4,352	5,584	3,008	2,304	3,456	8,000	6,592	7,168	5,248	5,056	6,720
Heated 20 min. at 56°C.	4,736	3,328	3,136	2,560	3,136	7,424	8,064	31,100	52,000	Innumerable	Innumerable
58°C.	4,480	3,200	2,560	3,136	3,392	8,064	9,600	48,900	Innumerable	"	"
60°C.	4,160	3,776	2,624	3,072	3,264	7,616	13,760	86,000	"	"	"
62°C.	4,480	3,072	2,626	2,944	3,392	8,000	17,920	Innumerable	"	"	"
65°C.	4,608	3,072	2,368	2,880	115,200	7,268	40,300	"	"		
70°C.	4,608	3,072	3,328	3,072	115,200						
80°C.	3,264	46,000	Innumerable	Innumerable							
Boiled 5 min.	4,352	57,600									

milk into the blood.   Ehrlich and Wassermann[10] noted that the milk contained from 1/15 to 1/30 as much antitoxin as the blood serum. Osborne and Wakeman[11] by analysis obtained 0.2 gm. of lactoglobulin per liter of milk.   Crowther and Raistrick[12] and Wells and Osborne[13] were unable to distinguish lactoglobulin from serum globulin by chemical or anaphylactic methods.   Howe[14] has shown that the blood of 30 months old cows contains on an average 5.06 gm. of globulin per liter.   If, then, the dilute products of blood serum are responsible for inhibition, a medium containing about 1/25 part of fresh blood serum should prove inhibitory.   That such is not the case is brought out in the following experiment.

Experiment 6.—The following medium was devised as a substitute for milk. Casein, 2.5 gm.; N/20 NaOH, 40 cc.; 0.9 per cent NaCl solution, 60 cc.; lactose, 5 gm.   Autoclaved for 15 minutes at 15 pounds pressure.   The reaction was pH 7.4.   To 25 cc. of the above 1 cc. of sterile fresh serum from Cow 1 was added. This was distributed into small tubes and inoculated with the streptococcus.   As a control procedure the medium which contained no serum was also inoculated with the culture.   Plates were prepared from both series at once and after 2, 4, 6, and 8 hours incubation.   As far as we could determine, growth took place to about the same degree in the medium containing serum as in the one which did not. There was an increase during the first 2 hours and a steady, though not rapid, increase thereafter.

It is true that an amount of serum proportionate to the globulin content of normal milk is insufficient to inhibit the growth of the non-hemolytic streptococcus in a relatively poor synthetic medium.

Rosenau has commented on the relative specificity of the reaction in milk since one of his samples restrained the typhoid bacillus and staphylococcus but not Parathyphoid A and B.   In the sense that Rosenau used the term "specific" it is probably true, since in our hands milk which would restrain the streptococcus had little effect on one strain of *B. coli* and only a moderate restraining influence on *B. bovisepticus.*

[10] Ehrlich, P., and Wassermann, A., *Z. Hyg. u. Infectionskrankh.,* 1894, xviii, 239.

[11] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.,* 1918, xxxiii, 7.

[12] Crowther, C., and Raistrick, H., *Biochem. J.,* 1916, x, 434.

[13] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.,* 1921, xxix, 200.

[14] Howe, P. E., *J. Biol. Chem.,* 1922, liii, 479.

Thus far there has been no evidence submitted to show whether the multiplication of one species of organism will absorb the inhibitory substance for another. With this in view the following experiment was devised.

Experiment 7.—Milk from Cow 13 was collected as usual and after centrifuging was divided into lots of 10 cc. Lot 1 was refrigerated at once; Lot 2 was heated to 62°C.; to each 10 cc. of Lot 3 the growth from 5 cc. of an 18 hour broth culture of the streptococcus was added; and Lot 4 was treated in the same way except that each 10 cc. received the growth from 7.5 cc. of broth culture of *B. boviseplicus.* Another portion of the milk was boiled for 5 minutes. The tube heated at 62°C. for 20 minutes and the tubes to which the cultures were added were incubated at 38°C. for 6 hours. The tubes inoculated with the cultures were then heated at

TABLE VI.

The Effect of Absorption with Streptococci and B. boviseplicus on the Inhibitory Substance in Milk.

	Streptococci present				
	At once	After 2 hrs	After 4 hrs	After 6 hrs.	After 8 hrs.
Raw milk, control	2,752	2,688	2,432	2,304	3,264
Milk heated at 62°C.	2,752	3,008	2,688	2,120	2,814
Absorbed with Streptococcus 55	3,200	4,736	Innumerable	Innumerable	Innumerable
Absorbed with *B. boviseplicus*	2,816	3,264	14,848	"	"
Boiled milk control		2,752	29,952	Innumerable	"	"

62°C. for 20 minutes. Exposure at this temperature was sufficient to kill the organisms but not inactivate the inhibitory property. It was necessary to adjust the reaction to the original pH of the milk of Lots 3 and 4 with N/Na_2CO_3. The effect of the absorption and heat was then tested in the usual manner after the addition of the mastitis streptococcus. The results are seen in Table VI.

Under the conditions it is apparent that the growth of one species of organism will absorb the inhibitory principle for another. It was not possible to completely absorb the substance, it is true, but the fact that for this purpose the strain of *B. boviseplicus* was about as efficient as the streptococcus argues that the substance is not specific for only the streptococcus.

We supposed from the frequent reference by other workers to fresh milk that this bactericidal substance deteriorated rapidly, even when kept at favorable temperatures.   On several occasions we tested milk that had been stored at 6°C. for 2 or 3 days and found the efficiency of the inhibitory element to be unimpaired.   After 10 days we failed to find any difference.   After refrigeration for 27 days the milk was still capable of inhibiting the growth of the streptococcus for 4 hours with slow growth from this time onward.   Originally it would inhibit 8 hours.   The sample held for a period of 2 months contained a large number of putrefactive organisms and could not be tested.

DISCUSSION.

It is apparent that there is present in the milk of all the cows examined a substance which will inhibit the growth of mastitis streptococci for a definite period.   The concentration of the inhibitory substance varies in the secretion of different animals.   It may be as concentrated in a young cow early in its first lactation period as in an older, more resistant animal which has been repeatedly exposed to infection with streptococci.   It is equally true that artificial immunization with killed cultures did not lead to an appreciable increase of the inhibiting agent in the milk.   The inference is that the substance is a natural one since it is not increased by exposure to streptococcic infection or artificial immunization and because it is not specific for a single organism.   The fact that the milk of young cows early in the first lactation period contains the substance in about the same amount as the older animals argues that it is inherent.

Whether or not such a substance under the present conditions of dairying may be regarded as of much value in protecting the udder against the rapid multiplication of bacteria which have gained entrance is open to question.   However, under the most favorable conditions it is possible that a few organisms which have recently gained access to the udder might be prevented from multiplying until they are flushed out at the next milking.   It may be that the substance is more potent than we suspect, since many opportunities are available for the entrance of bacteria into the teat canal although in the main the flora of the udder is limited to relatively few species and these

species have become well adapted to the environment and the inhibitory influence of this substance.

Under more natural conditions the substance might have considerable influence in protecting the udder. From the experiments the inhibition of growth of the mastitis streptococcus during the first 4, 6, or 8 hours was strong. Under natural conditions the calf would empty the udder at intervals which correspond to these periods. The inhibiting agent would act to prevent bacterial multiplication and thus insure milk of low bacterial content for the calf. The lengthening of the period between milking to 12 hours under the usual conditions of dairying has more or less rendered the substance inoperative.

Although we have no data directly bearing on the origin of the substance, by inference it seems possible to localize its source. Rosenau's contention that the diminution is due to agglutination is probably true for the organisms he tested. Our technique seems to rule out agglutinin, especially in view of the microorganism employed. The blood contains only weak agglutinin for the streptococcus, which is not easily agglutinated, and clumps could not be detected on microscopic examination of the milk.

If the substance is of blood origin the inhibitory substance should be greatly increased in the whey since with rennet coagulation there is a considerable concentration of the blood proteins, as shown by serum precipitin tests of the whey.

The view that the substance is "alexin" from the blood is not supported by our observations. Thus, in the normal udder there is only a slight interchange of blood proteins from the circulation to the milk and the concentration is too low to be a serious factor. This fact is borne out by the experiment in which fresh blood serum was added in the proportion of 1:25 to a special medium containing casein and lactose without inhibiting the growth of the streptococcus. The alexin from the blood of cows, like that of most mammals, is inactivated at a temperature of 56°C. for 20 minutes, while milk containing the inhibitory substance may be heated for the same period at 62°C. without impairing its inhibitory properties. It is not completely inactivated at 65° and 70°C. In this respect, then, there is a distinct difference between the substance in the milk and that of the blood. We infer from these facts that the substance originates in the udder.

It however resembles alexin in that it deteriorates slowly even at a temperature of 6°C.

Hanssen's contention that the substance is an oxidizing enzyme must be given consideration. He noted that the inhibition was more marked in the cow on pasture than in the milk of the same cow during the winter. From this he inferred that the substances originated in the food. The cows in our observations were fed about the same ration throughout the year.

Our experiments further indicate that the action of the substance is not specific, since by incubating raw milk with one organism we were able to absorb the inhibiting agent for another.

SUMMARY.

The bactericidal activity of fresh raw milk from a number of cows has been tested with the non-hemolytic mastitis streptococcus. By using this organism and other means we were able to rule out the action of agglutinin. The milk of all cows examined inhibited the growth of the streptococcus for definite periods. The length of the inhibition period varied; the milk from some cows prevented growth for 8 hours, that of others for only 4 or 6 hours. The inhibitory action may be as strong in the milk of a young cow in its first lactation period as in that of an old cow known to be resistant to udder infection. It is possible to absorb the streptococcus inhibitory substance by first inoculating the milk with *B. bovisepticus*. We were unable to show that the substance was increased by artificial immunization of cows with the streptococcus.

Whey obtained by the action of sterile rennet solution inhibited the growth of the streptococcus to about the same extent as the milk from which it was obtained.

We infer that the substance originates in the udder since it differs from blood alexin in its resistance to heat, it is not increased in the whey although the blood proteins are more concentrated, and it is not increased in the milk when the cows are artificially immunized or repeatedly exposed to natural infection.

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THE RELATION BETWEEN INVASION OF THE DIGESTIVE TRACT BY PARATYPHOID BACILLI AND DISEASE.

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The large group comprising the colon, paratyphoid, and typhoid bacilli have certain characters in common which have led most students of this group to assume a close genetic relationship. The production of disease by members of the colon group resides in a capacity to multiply in the lower small intestines with coincident production of a toxin or toxins. These are absorbed and produce certain effects leading to extreme congestion of the capillary system and hemorrhage especially marked in the kidneys. The capacity to produce disease among the highly parasitic types of the paratyphoid group, such as the hog cholera bacillus, consists in the power to penetrate the mucosa, lodge and multiply in certain viscera, chiefly spleen, lymphoid tissue of the intestines, and liver, where a toxin similar to that produced by *B. coli* comes into intimate contact with certain vulnerable cells. Between these extremes we may assume that there exist many varieties either approaching one or the other of these types or so balanced that multiplication in the intestinal tube and the invasion of the tissues and multiplication therein both contribute to the disease.

The extensive literature dealing with epidemics of food poisoning and with the clinical paratyphoid disease in man serves to trace the different types of disease. Some are evidently due chiefly to gastrointestinal intoxication; others to invasion of the body, in which respect they approximate the behavior of the typhoid bacillus. If the above grading of the colon-typhoid group is true, it follows that the invasion of the digestive tract in itself may or may not be followed by clinical manifestations. In the colon bacilli it would be necessary that they multiply abundantly in the tube itself in order to produce

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enough toxin; for the other extreme, that they penetrate the mucosa and find no resistance to temporary multiplication in the blood or lymph channels or even within certain cells. The immediate bearing of the disinfecting action of the stomach and its relation to penetration of bacteria through the mucosa of mouth and throat instead of the intestinal wall need not enter into the problem, nor the obvious fact that different host species will react differently when invaded by the same type of bacteria. Moreover, certain abnormal states of the digestive tract may modify bacterial behavior.

The general question here raised has an important bearing upon an understanding of vaccinations against this group of bacteria. In the case of human typhoid the subcutaneous injection of bacilli killed by heat has the support of statistics as to its efficiency. The injection of dead bacteria tends to protect the body whose digestive tract is invaded by typhoid bacilli. Does the typhoid bacillus multiply in the lower small intestine after introduction or only later in the disease after it has been discharged through the bile and from local ulcers into a tube whose protective mechanism has been disorganized by the disease process? Are there races of typhoid, some capable of multiplying in the intestines at the start, others promptly invasive? The vaccine probably has no effect on bacilli in the tube but only after they have penetrated the mucosa. Disease might conceivably be produced by races simulating the colon type even after vaccinal protection.

In approaching questions of this kind experimentally, the nearer our experiment to natural conditions, as far as known, the nearer our results approach the solution of the natural or practical problem. At the same time any other combination of experimental procedures may assist in analyzing the problem and thus provide material for a future synthesis approaching practical conditions.

The experiments to be reported were begun in 1921 before the extensive researches on mouse typhoid by Flexner and Amoss, Topley, and Webster[1] had appeared, and were closed in August, 1923. They are incomplete but they furnish some data of value in the final

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[1] For a good bibliography of this work up to 1926 see Lockhart, L. P., *J. Hyg.*, 1926, xxv, 50.

formulation of factors governing the rise and fall of mortality in this group of diseases.

Methods.

To bring the problem within the field of experiment, the hog cholera bacillus and the white mouse were chosen. The susceptibility of gray mice to this bacillus had been studied by one of us in 1885. At this time the breeding of white mice for experimental purposes had scarcely begun. The white and the gray mouse have about the same degree of susceptibility towards the hog cholera bacillus. The gray mouse, however, as usually obtained in traps is a more variable subject often showing lesions of the kidneys and other organs due to various parasites and possibly poisons. The strain of the hog cholera bacillus used in the following experiments had been under cultivation for about 6 months when the experiments were begun.[2]

It is obvious that this relationship of hog cholera to white mice is artificial and that an analysis of it can cover only a narrow portion of the entire field. The minimum fatal dose, given subcutaneously, varied considerably from case to case. An 18 to 24 hour bouillon culture in which the final clouding is fairly constant was used in all cases. In 1921 the surely fatal dose was about 0.05 cc. Nearly 50 per cent succumbed when 0.02 cc. was injected. A small number died from doses down to 0.002 cc. Death resulting from a minimum dose usually occurred on the 7th to 9th day, rarely later. Doses representing multiples of the minimum dose killed within 4 days. In later experiments (1922-23) the minimum fatal dose was evidently somewhat larger. In an animal as small as the mouse the skill with which the infecting dose is deposited in the subcutis in each mouse, not too deep into the muscular tissue or even the peritoneal cavity nor into the skin itself, probably determines in part the death rate. No attempt was made to increase by passages the virulence of the strain used. Each experiment was started with the stock culture grown on sloped agar and kept at about 38-40°F. in the refrigerator after each monthly transfer had been incubated 1 or 2 days.

EXPERIMENTAL.

From the 50 or more mice used in preliminary trials which received subcutaneous doses of hog cholera bacilli and survived, the injected bacilli were demonstrated in the spleens in all cases up to the 75th day, the longest period tested. The spleens, usually larger than in the normal condition, were torn into three or four pieces which were placed in tubes of sloped agar. There was evident a gradual diminution in the number of colonies as the period between inoculation and

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[2] This strain was kindly sent by Dr. Dimock of the University of Kentucky.

killing widened. In mice killed when sick in the early days up to the 10th the colonies were numerous. In those active and apparently well when chloroformed they were scarce even in bits of spleen as large as split peas.

After considerable experimentation with one lot of white mice, a

TABLE I.

Mouse No.	Subcutaneous dose	Died (d) or killed (k.)	Cultures (spleen)	Remarks
	cc.			
1	0.05	d. 7 days	Many colonies	
2	0.05	d. 9 "	" "	
3	0.01	k. 25 "	Few "	Some pneumonic foci
4	0.01	k. 25 "	" "	" " "
5	0.01	d. 9 "	Many "	
6	0.01	d. 10 "	" "	

TABLE II.

Mouse No.	Dose fed	Died (d.) or killed (k.)	Spleen cultures
	cc.		
1	6	d. 11 days	Many colonies
2	6	k. 25 "	Few "
3	6	d. 13 "	100 "
4	6	k. 15 "	Growth only in condensation water
5	6	d. 7 "	Many colonies
6	6	k. 26 "	1 colony
7	6	d. 11 "	Many colonies
8	6	k. 15 "	" "

pneumonia appeared which entered as a disturbing factor as Table I indicates.

It will be noted that of the four mice receiving 0.01 cc. subcutaneously the two affected with pneumonia survived. Another lot of mice was raised from a small group obtained from an isolated colony and this remained free from the pneumonia.

Inasmuch as the problem before us concerned the relation of the digestive tract to infection, feeding of cultures was resorted to.

An 18 to 24 hour bouillon culture was used in a definite amount to moisten a small piece of dog biscuit which was placed in a solid watch-glass in the container, a battery jar with wire mesh cover. The mice were of the nearly uniform age of 2 months when used. They were all of the new lot which was free from mouse typhoid and pneumonia. The food consisted of corn, oats, and dog biscuit, with a constant supply of water. Before infecting the mice they were fasted overnight.

TABLE III.

New Stock of Mice.

Mouse No.	Bouillon culture fed	Died (d.) or killed (k.)	Spleen cultures	Remarks
	cc.			
47	0.2	d. 7 days	Many colonies	
48	0.5	d. 7½ "	" "	Cysticerci in liver*
49	0.5	d. 6 "	" "	" " "
50	1.0	d. 7 "	" "	
51	2.0	k. 9 "	" "	" " "
				Very sick when killed
52	3.0	d. 7 "	" "	

Old Stock.

59	0.2	k. 28 days	+－－**	
60	0.5	d. 10 "	Many colonies	
61	0.5	k. 28 "	+++	
62	1.0	d. 9 "	Many colonies	
63	2.0	k. 34 "	+++	
64	3.0	k. 34 "	++－	

* Only the original stock of new mice were infested. The offspring were free.
** 3 pieces of spleen in 3 tubes of agar. 2 remained sterile.

As a rule the food placed before them was entirely consumed within 18 to 24 hours. In the earlier experiments two mice were fed in a jar and kept together. The irregular results pointed to the possibility that one of the two mice had eaten the bulk of the infected food. This surmise is borne out by the experiment shown in Table II.

It will be seen from the table that only one of every pair died. The other must have obtained some of the food, for spleen cultures were positive 15 to 26 days after the feeding when the surviving, still

active mouse of every pair was chloroformed. Thereafter only one mouse was fed in a jar.

Before giving up the old stock of mice a test was made of their resistance to the hog cholera bacillus as compared with that of the new stock. Six mice each were fed as described and each kept by itself in a jar.

It will be noted that five out of six of the new stock died. The sixth was very sick when chloroformed and would have probably died. The tapeworm infection of the original mice of the new stock, as observed in other mice of this lot, did not influence the outcome. Of the old stock only two out of six died. The remaining four were killed after 28 to 34 days. It will also be observed that the result did not go parallel with the dose fed.

TABLE IV.

Mouse No.	Dose fed	Died (d.) or killed (k.)	Spleen cultures
	cc.		
17	7	d. 6 days	Many colonies
18	Control	k. 25 "	Sterile
19	7	k. 19 "	A few colonies
20	Control	k. 25 "	Sterile
21	7	d. 7 "	20 colonies
22	Control	k. 25 "	Sterile
23	7	d. 6 "	Many colonies
24	Control	k. 31 "	Sterile

As a preliminary test the degree of infectivity of mice fed with hog cholera bacilli to other mice associated with them was tried in the following way.

Mice were distributed one in a jar and, after withholding food, fed with dog bread moistened with a fresh bouillon culture. A very large dose was given the mice purposely to bring about as heavy a discharge of bacilli as possible. On the following day the fed mice were removed to clean jars and a fresh mouse added to each jar. All but one of the fed mice died, with the usual postmortem appearances and positive spleen cultures. The one survivor was killed on the 19th day. The spleen culture was positive. The exposed mice remained active and were killed 25 to 31 days after the beginning of the exposure. The spleen cultures made with large bits of tissue remained sterile.

In a second test, after feeding, the fed and the control mice were placed together in a wire cage. They were killed in pairs after 4, 5, 6, and 8 days respectively. In none of the four exposed mice were hog cholera bacilli detected, in the spleen, abdominal cavity, duodenum, ileum, and feces. Thus far the experiments indicated in all cases an invasion of the body after feeding cultures as shown by the presence of hog cholera bacilli in the spleen in all survivors for an indefinite period up to $2\frac{1}{2}$ months. Contacts with mice fed large doses did not carry bacilli in their spleens or digestive tracts.

It was next decided to analyze in some detail the condition of the digestive tract following feeding.

To ensure uniformity the mice used were from the new stock bred originally from only a small number of individuals. They were all about 2 months old and weighed from 17 to 23 gm. The diet and method of feeding remained the same. The culture was fed on a small piece of dog biscuit.

Cultures were made from certain regions of the alimentary tract and in some experiments the material was weighed after suspension in a definite amount of bouillon and a calibrated loop of this rubbed over agar plates in order that some rough conception of the numbers of hog cholera bacilli present might be gained. Cultures from the intestinal contents required careful manipulation since hog cholera bacilli were present in the abdominal cavity of most, if not all, infected mice. The portion of intestine desired was cut out and removed to a sterile petri dish. With sterile instruments the tube was cut longitudinally. In most cases the walls curled outward, exposing the contents, so that they could be removed with forceps without difficulty. The contents were suspended in bouillon. Feces were removed by holding the living mouse as if for injection and gently rubbing the abdomen with the finger. When feces were ejected, they were taken with sterile forceps and put into bouillon and shaken. From these shaken suspensions of intestinal contents and feces, plates were made directly with one loopful for each plate. The suspension was then kept at room temperature overnight. If the hog cholera organism did not appear on the first set of plates, the next day duplicate ones with malachite green were made from the incubated material. Two agar plates were made from each specimen, one contained 0.3 cc. of a 1 per cent aqueous solution of malachite green per 10 cc. of agar. The plain agar plate made possible a general survey of the intestinal flora of each mouse, and the malachite green agar inhibited growth of nearly all but the hog cholera bacillus. Occasionally there was found on the plates an organism which grew like the hog cholera bacillus. A large bubble of gas in lactose broth and negative agglutination tests with a specific rabbit serum sufficed to distinguish this organism from the hog cholera bacillus.

The first series of tests to be described determined roughly the number of organisms found in the stomach after the beginning of feeding and the survival time. The 18 to 24 hour bouillon culture

TABLE V.

Bacteria in Stomach of Fed Mice.

Mouse No.	Killed after feeding	Bacteria in 0.15 gm. stomach contents	Calculated total in stomach contents	Stomach contents actually plated
			First Test	
	hrs.	millions	millions	gm.
113	2	65	261	0.00005
114	3	195	716	0.00005
115	4	15	37	0.00005
116	5	68	157	0.00005
117	26	0	0	0.00005
118	29	0	0	0.00005
119	48	0	0	0.0005
			Second Test	
120	2	233	570	
121	3	74	294	
122	5	83	168	
123	6	77	116	
124	8	231	231	
125	18	16	28	
126	24	0	0	Incubated contents also negative
127	51	0	0	
			Third Test	
		thousands	thousands	
168	18	24	151	0.0009
169	20	91	427	0.0009
170	22	<1	<1	0.0009
171	24	0	0	Incubated contents also negative

was added in amounts of 0.5 cc. to a gm. of dog biscuit and each mouse fed separately in a mouse jar. Three separate tests were made. In the two first the mice were left in the jars in which they

had been fed. In the third they were removed to clean jars and fresh food given after 17 hours.

The results agree in demonstrating the presence of large numbers of hog cholera bacilli during the early hours after feeding. A decline occurs about the 18th hour and in 24 hours the fed bacilli are no longer detected in the contents.

Hydrogen ion determinations of stomach contents, according to Brown's drop method,[3] were made on three mice killed $1\frac{1}{4}$ hours after a meal of corn and oats. The pH readings were 5.4, 5.6, and 5.8.

In a second series of experiments comprising three lots of mice, 10, 10, and 12 in number, respectively, an attempt was made to follow the fed bacteria beyond the stomach into the lower digestive tract, the peritoneal cavity, and the spleen.

In the first series the mice were fed each 0.5 cc. of bouillon added to 3 gm. of dog biscuit. The 1st mouse was killed 24 hours after the beginning of the meal, and one on each succeeding day, except the 5th day. The 10th mouse was therefore killed on the 11th day after feeding. Spleen cultures were positive in all but the 1st mouse. Cultures from the peritoneal cavity were positive in all but the 2nd and 4th mouse. The fed bacilli were obtained on plain and malachite green agar plates from contents of the ileum in all cases; in the 4th and 7th mouse, however, only after incubating the contents in bouillon overnight. On the plain agar plates the fed bacilli were overgrown by colon bacilli in the 3rd, 6th, and 9th mouse.

In the second and the third series the examination was extended to duodenal contents and feces and to the 21st day after feeding.

The results agree with those of the first series. Two mice died in the third series after 9 and 11 days. One mouse in the second series was negative throughout. In addition to these series, two mice were fed and kept 28 days. The spleen cultures were positive. Plates from duodenum, cecum, and feces contained colonies of hog cholera bacilli in both mice. A few bacilli were found in contents of ileum of one only.

The prompt invasion of the peritoneal cavity and spleen by way of the digestive tract suggested a final experiment in which the reverse

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[3] Brown, J. H., *J. Lab. and Clin. Med.*, 1923–24, ix, 239.

route was to be traced. Three small groups of mice received sub-
cutaneously 0.02 cc. of a 24 hour bouillon culture in 0.1 cc. fluid.
They were chloroformed up to 41 days after injection and the con-
tents of ileum, cecum, colon, and discharged feces spread on plain
and malachite green agar plates.

TABLE VI, *a*.

Killed, days after feeding	Cultures			No. of organisms per 0.016 gm. of ileum contents
	Spleen	Duodenum	Feces	
1	—	—	+	11,600
2	+	—	—	2,227
3	+	+	+	6,032
4	+	—	+	39,771
6	—	—	—	0
7	+	+	+	18,560
8	+	+	+	185,600
9	+	+	+	659,569
10	+	—	+	2,735
11	+	—	—	0***

TABLE VI, *b*.

11	+	+	+	5,290
12	+	+	+	3,648
13	+	—	+	214
14	+	—	+	4,690
15	+	+	+	21,458
16	+	—	—	0
18	+	—	+	0***
19	+	—	+	426
20	+	—	+	95
21	+	+	+	0***

*** Recovered on plates after incubating material in bouillon overnight.

Among the first group of eight, one died within 24 hours of some
undetermined cause. Invasion of the intestinal tract was evident
after 3, 7, and 15 days. Mice killed after 2, 5, and 21 days respectively
were negative in this series. In the second group of eleven mice,
five died. These mice were smaller than those used hitherto. All

but one of the six survivors were sick when killed. In all the intestinal tract contained hog cholera bacilli. The third group of

TABLE VII.

Killed, days after injection	Cultures				Condition of mouse when killed
	Ileum	Cecum	Large intestine	Feces	
First Group					
2	—	—	—	—	Well
3	+	—	—	—	"
5	—	—	—	—	Sick
7	+	+	+	—	"
8	—	—	—	+	Well
15	+	+	+	+	"
21	—	—	—	—	"
Second Group					
7	+	+	+	+	Sick
8	+	+	+	+	Very sick
9	+	+	+	—	" "
10	+	+	+	+	" "
13	+	+	+	+	" "
16	+	+	+	+	Well
Third Group					
12	—	—	—	—	Well
13	+	—	—	—	Sick
14	+	+	—	—	Slightly ill
16	+	+	+	+	Well
17	+	—	+	+	"
19	—	—	+	—	?
21	+	+	+	+	Well
37	—	—	+	—	"
39	—	—	—	—	"
41	+	—	+	+	"

twelve mice was more resistant. Only two died in 4 and 9 days, respectively. In two mice killed after 12 and 39 days respectively injected bacteria were not recovered.

A control experiment was made to eliminate the chance that the injected culture dilution might ooze out locally, be lapped by the mouse, and introduced into the digestive tract directly. During the injection the needle was inserted under the skin about 1 cm. The skin was subsequently washed with 95 per cent alcohol, dried, collodion applied to the punctured area and allowed to dry before the mouse was caged. Six mice received 0.02 cc. culture fluid in 0.1 cc. bouillon dilution. Two died after 6 and 7 days, respectively. The rest were killed after 7, 8, 9, and 12 days. All had the injected bacteria in contents of cecum, colon, and in the feces. In all but one (9th day) bacteria were found also in the ileum.

<div align="center">DISCUSSION.</div>

Before we attempt an interpretation of the experimental work, a brief statement of the lesions following the feeding or injection of hog cholera bacilli in mice may be helpful. Following a minimum fatal dose injected subcutaneously mice die in 7 to 9 days, rarely later. If the mouse is chloroformed when manifestly sick, the mucosa of the intestinal tract will be found with nearly intact epithelium. The gross lesions observed involve liver and spleen. Examination of fixed and hardened tissue show involvement of lymph nodes, mesenteries, and peritoneum generally and to a certain degree of the mucosa of the large and more rarely of the small intestine.

The lesions of the liver are the most constant and characteristic. Hog cholera bacilli just isolated from swine produce sharply defined necroses, 1 to 2 mm. in diameter.[4] These rarely appear following inoculation with strains under prolonged cultivation. The lesions always encountered comprise the presence of cells of endothelial type in the sinusoids. The entire liver is involved in this condition. The sinusoids may contain a few cells not encroaching on the liver tissue, or many closely packed, tubercle-like, and causing disappearance of surrounding liver cells. The spleen is always large, dark red, firm. The tissue changes differ according to the severity of the induced disease. In the fatal cases, there is much hemorrhage or at least marked distention of the pulp vessels and the focal accumulation of endothelial cells. In recovering cases the lymph follicles are large. The mesenteries are broadened by cell accumulations of endothelial and lymphoid type. The lymph nodes are enlarged and the changes like those going on in the spleen.

[4] Boxmeyer, C. H., *J. Med. Research*, 1903, ix, 146.

The intestines interest us more particularly in view of the special problem of this communication. In the presumably fatal cases there may be found here and there in the sections of the large intestine accumulations of endothelial cells in the mucosa, forcing the crypts apart or else causing them to disappear. The infiltrated zones are as a rule still covered with epithelium. Actual ulcers were very rare in our cases. The large intestine is the dominant seat of such infiltrations, although they may also be found in the small intestine. There is no evidence that Peyer's patches or lymphoid follicles are involved to any degree. They are not the necessary loci for the changes. The feeding of large doses does not lead to diffuse injury to the surface epithelium or the free portions of the villi such as might be looked for when a toxin is freely discharged from multiplying bacteria and such as we note when poisons have been ingested. Nor do we observe the severe congestions often leading to hemorrhages which are associated with unrestricted multiplication of *B. coli* in the small intestine of calves.

The hog cholera organism is evidently of a more highly adapted, parasitic type than the cultures used by Webster and Topley. The disease follows subcutaneous inoculation and intraperitoneal injections are unnecessary. Deaths earlier than 4 days are rare. They tend to occur between the 7th and 9th day more or less independently of the dose within limits. These facts indicate a greater penetration and resistance to destruction and a more stabilized march of the disease process. Acute intoxication and early death is produced only by many multiples of the surely fatal dose.

The main outcome of these studies is the demonstration of the readiness with which hog cholera bacilli pass into the blood and lymph circulation after ingestion in food and the uniformity with which they make their appearance in the intestinal tract after subcutaneous injection in surviving mice. The second point to be noted is the persistence of the fed or injected bacilli in the spleen, even though in very small numbers. The prompt dissemination may be ascribed in part at least to the small size of the animal, the delicate tissues, and the short distances to be travelled. On the other hand, the universal invasion without marked illness or death suggests that, to compensate for this early entrance, the mouse is endowed with a high degree of

fundamental resistance, or natural immunity, which represses multiplication. This is evident when the mouse is compared with the rabbit which succumbs to the minimum lethal dose of the mouse.

Clinical disease manifested in the mouse by crouching on top of the bedding with staring coat and somnolent behavior is not followed by recovery. If the mouse is killed at this time, many bacteria are found in the spleen, and the obvious inference is that disease is associated with multiplication of the bacilli in the viscera and when this has reached a certain momentum reversal of the process and recovery are no longer possible. The relation of feeding to clinical disease and death is not cleared up by the experiments reported. As we trace the fed bacteria down, we note during the first hours large numbers in the stomach contents which completely disappear within 24 hours. Some have probably passed into the duodenum. The numbers found in the ileum may represent those passed into the small intestine from the stomach and they may be the residuum of a process of destruction and multiplication going on side by side. 2 weeks after feeding there are no bacilli found or else only a few in this section of the small intestine. Their discharge in the formed feces goes on parallel with their presence in the digestive tract and spleen. On the whole the assumption is warranted that clinical disease depends on the numbers that penetrate into the body proper and multiply there. The conditions favoring penetration are unknown. The passage of bacilli outward may be due in part to the causes operating in favoring penetration inward, but in view of the uniform and diffuse involvement of the liver it is more probable that they are discharged in the bile. Lesions of the intestinal tract characterized by ulceration of the mucosa are very rare and they may play a very subordinate part in the outward discharge.

If multiplication in and consequent intoxication from the alimentary tract were factors when large doses are fed, we should expect the appearance of symptoms within 2 days, allowing for mass destruction in the stomach during the 1st day. This does not occur. The evidence points to penetration and subsequent multiplication reaching a climax within a week. The marked irregularity in the effects of small and large doses of hog cholera bacilli fed indicates that the presence of such bacilli in the intestinal tract of mice does not imply a

corresponding absorption into the body proper, although such absorption takes place since bacilli are always detected in the spleen later on. A large dose fed may evidently be followed by slight absorption and if we assume that it is the numbers absorbed that determine the clinical manifestations, the marked absence of agree-, ment between the doses fed and active disease is satisfactorily explained. The concept of absorption includes both penetration into the body and capacity to multiply. Here the individual immunity of the host must come into play. In these studies no attention has been paid to the possible relation of the minute nematodes of the cecum to the appearance of disease after feeding, nor were the intestinal contents examined for the overmultiplication of flagellates, occasionally observed in mice.

The passage of bacteria into the system from the digestive tract may stand in some causal relation to motility. To the writers' knowledge attention has not been called to this possible relationship. This function is remarkably persistent in the paratyphoid group under cultivation and is possessed by all pathogenic members of the group. In fact its persistence in bouillon cultures over 24 hours old, in the sense that it is shared by most organisms in the culture, is presumptive evidence that the organism belongs to the above group rather than to *B. coli*. Its persistence in a pathogenic group strongly argues for its usefulness and in the penetration of the normal mucosa it may explain the invasiveness of the hog cholera bacillus as compared with *B. coli*. After its entry into the system in small numbers by this method even in the presence of large numbers in the alimentary tract, a slight increase in resistance may suppress multiplication, whereas the introduction parenterally even of small doses may soon exhaust the normal, antibacterial forces. Experiments to test the significance of motility are under way.

CONCLUSIONS.

Hog cholera bacilli fed to mice disappear from the stomach within 24 hours, but remain and perhaps multiply in the ileum for at least several weeks. They promptly penetrate the mucosa and may be found in the spleen. Bacilli introduced subcutaneously quickly pass

into the intestinal tract where they may be found for some weeks. Infected mice may harbor bacilli in the spleen for several months.

Mice possess a relatively high degree of natural resistance towards hog cholera bacilli which gives way to large doses. Disease is probably the result of the invasion of the viscera from the digestive tract following feeding, but the relation between the dose fed and the numbers penetrating the mucosa is a variable one and the conditions favoring such invasion not determined. Contact with mice discharging bacilli failed to cause recognizable invasion of the digestive tract or the viscera.

STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

I. Report of a Natural Outbreak of Paratyphoid in a Guinea Pig Population.

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During the summer of 1924 a spontaneous outbreak of paratyphoid occurred among the guinea pigs maintained for breeding purposes and for stock. Throughout a 5 year period prior to the outbreak the guinea pig population had been conspicuously free from disease. No animals had been introduced from outside sources during this time. The stock represented a rather homogeneous population but not a vigorously inbred one. Inasmuch as the outbreak was of spontaneous origin in a uniform population known to have been free from disease for a period of years, it was deemed worthy of study. The infection was followed through the active stage of the epidemic* and an endemic stage which subsequently ensued. Particular attention was paid to the latter stage in an endeavor to ascertain some of the factors accountable for the maintenance of the infection in the form of sporadic cases and carriers. The present paper will deal only with the course of the outbreak, the gross pathology of the disease, and the bacteriology of the causal organism. The factors which seemed significant in determining the maintenance of the infection through the endemic period will be discussed in a second paper.

Numerous references to spontaneous paratyphoid in the guinea pig may be found in the literature. The disease appears to have been recognized as early as 1884 and subsequently called bacillary pseudotuberculosis of the guinea pig

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* We are indebted to Dr. Laura Florence for making autopsies and cultures of guinea pigs during July and August of 1924.

by Eberth (1) in 1885. T. Smith and J. R. Stewart (2) in 1897 reported a single
case of pseudotuberculosis in a guinea pig and were able to isolate an organism
from the spleen which was culturally related to the hog cholera bacillus and to
B. typhi murium of Loeffler. Wherry (3) in 1908, investigating the efficacy of the
"Azoa" virus for the extermination of rats, encountered the disease in his guinea
pig stock. He isolated a bacterium apparently identical with that found by
Smith and Stewart. He named it *B. cholera caviæ* but later changed it to *B.
pestis caviæ*. On the basis of direct agglutination he judged it to be identical with
the "Azoa" bacterium. It showed only remote relationship to a human strain of
B. enteritidis. Howell and Schultz (4) in 1922 reported a serious outbreak of
guinea pig paratyphoid with high mortality. They isolated a number of different
strains of *B. paratyphi*. All of the strains were distinct from *B. paratyphi* a and
b, *B. enteritidis*, and *B. suipestifer*. Thomas (5) in 1924 encountered spontaneous
infection in a small series of experimental guinea pigs. By agglutination tests he
identified the organism isolated from fatal cases as either *B. enteritidis* or *B.
paratyphi* of animal origin. He described the gross and microscopic pathology of
the disease. A detailed summary of the literature pertaining to guinea pig para-
typhoid up to 1916 is given by Holman (6) in a survey of spontaneous infections
of the guinea pig. Paratyphoid has been studied in the mouse from the standpoint
of experimental epidemiology, and reference should be made to the extensive
researches of Webster (7), Topley (8), and their associates.

The guinea pigs comprising the population in which the outbreaks
of paratyphoid occurred are maintained in three groups. The first
group includes the breeding animals, divided into units of 5 sows
and 1 boar kept in single cages. At parturition each sow with her
young is removed to a separate cage and kept there until the young
guinea pigs are weaned, a period of about 2 weeks. This forms the
second group, which is maintained in the same room with the breeding
stock proper. The cages are of metal, well separated, and offer only
a remote possibility of direct communication. As soon as the young
guinea pigs are weaned the sow is returned to the original breeding
unit, the young separated as to sex and added to the stock benches.
Here are segregated young pigs of the same sex and approximately the
same age in groups of 20 to 30 individuals. They are the weaned,
stock guinea pigs for general laboratory use and constitute the third
group. They are maintained in a separate room from the breeders
and unweaned young. During the months of July and August, in
order to stop breeding approximately half of the boars are removed
from the breeding units to individual cages in a separate room. Such

replacements as become necessary among the breeders are made from young guinea pigs selected from the stock.

The epidemic began on the 14th of July, 1924, and continued for a period of 8 weeks. At that time the population was composed of 597 animals' distributed as follows: breeders 310, unweaned young 69, stock pigs 218. During the 8 week interval 104 individuals succumbed to the infection. Irrespective of groups the mortality rate for the period was approximately 17 per 100 of population. The distribution of deaths within the population was of some interest. The infection first appeared among the breeders which numbered, at the time, 250 sows and 60 boars. As previously stated, half of the boars had been removed from the breeding units during the summer, so there were actually only 30 males in the breeding room. During the 1st week 6 sows died from paratyphoid. The number of deaths

TABLE I.

Deaths per Week among Breeders during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
6	8	2	10	8 .	4	2	3

per week increased slightly, to 10 animals, during the 4th week and then declined. A total of 43 individuals from this group succumbed to the infection; all of them were sows. No deaths occurred among the boars exposed to infection in the breeding units nor among those isolated in individual cages. The number of deaths per week among the breeders during the 8 week period is given in Table I.

Exactly a week after the epidemic began the first deaths occurred among the stock pigs which numbered, at the time, 218 individuals. The number of deaths per week reached a peak during the 3rd week and then declined. There was a total of 50 deaths for the 8 week period. The number of deaths per week is given in Table II.

The appearance of the infection among the guinea pigs of the second group, sows with unweaned young, practically coincided with its appearance among the stock pigs. During the 8 week period a total of 11 deaths occurred among the 69 individuals which composed the

population of unweaned young at the start.   Data are incomplete
as to the number of sows with young during the 8 week interval.   1
sow succumbed to the infection.   The number of deaths per week is
given in Table III.

The active stage of the epidemic lasted 8 weeks and was followed
by an endemic period, characterized by sporadic deaths, which has
continued to the present time (May, 1926).   During the year 1925, a
total of 229 deaths from all causes occurred in the guinea pig popula-
tion.   Of that total, 91 animals showed sufficient evidence to warrant
the diagnosis of paratyphoid as the cause of death.   Those guinea
pigs that showed no evidence of specific infection were generally

TABLE II.

Deaths per Week among Stock Guinea Pigs during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
0	11	16	6	8	8	0	1

TABLE III.

Deaths per Week among Unweaned Young during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
0	2	0	1	3	5	0	0

young, unweaned individuals that died shortly after birth.   In most
cases they appeared to be individuals that had received insufficient
nourishment from the dam.   The total of non-specific deaths in-
cluded, in addition, a smaller number of stock guinea pigs and breeders
that showed a variety of abnormal conditions upon autopsy.

The distribution of specific deaths within the population during the
year 1925 was as follows: breeders, 23; stock guinea pigs, 28; un-
weaned young, 40.   Distributed as to sex there were 23 deaths among
males and 62 deaths among females.   Data were lacking in a few
instances.   The total number of deaths per month, the number of
deaths from paratyphoid, the percentage mortality from paratyphoid,
and the estimated population by months are given in Table IV.

The gross pathological manifestations of the disease, encountered at autopsy, varied considerably. The most constant lesion, the one which led to the early name of pseudotuberculosis, was found in the spleen. In typical cases the surface was studded with foci varying in size from minute white points, barely discernible, to large yellowish nodules projecting above the surface and measuring several millimeters in diameter. Focal changes were found in the depths of the splenic tissue as well as on the surface. In addition there was marked enlargement and congestion of the spleen. With more acute cases death

TABLE IV.

Total Deaths, Deaths from Paratyphoid, Percentage Mortality from Paratyphoid, and Population by Months during 1925.

Month	Population	Total deaths	Deaths from paratyphoid	Percentage mortality from paratyphoid
January........	856	8	3	0.35
February........	1,105	12	3	0.27
March..........	1,006	9	3	0.29
April..........	800	25	17	2.15
May...........	677	14	5	0.73
June..........	524	44	21	4.00
July...........	401	51	10	2.49
August........	460	8	5	1.08
September.......	438	27	16	3.65
October.........	500	7	5	1.00
November.......	540	9	3	0.55
December.......	581	15	0	0.00

resulted before focal changes had occurred and the spleen showed only enlargement and congestion. There were gradations between the two extremes marked by the presence of occasional foci. In several instances an exudative membrane partially covered the surface of the spleen.

The liver often showed foci varying in number and in size. At times there was marked congestion and at times fatty degeneration. In some instances the gall bladder was distended by a thin, purulent fluid. The intestinal tract, particularly the small intestine and cecum, showed congestion. In the cecum small, circumscribed areas of

hemorrhage were often present.   There was generally an involvement of Peyer's patches, which in the more acute cases were swollen and showed small gray points giving a stippled appearance.   In the less acute cases in which sufficient time had elapsed for the appearance of focal changes in other tissues, the same were generally discernible in Peyer's patches as distinct nodules, white or yellowish in color, and varying in number from one to sufficient to pack the entire patch. Lymphoid tissue in other localities, the mesenteric nodes and cervical nodes, often showed involvement varying from swelling with congestion to the presence, in addition, of foci.   Less constant changes noted in other organs were an anemic condition of the kidney and a patchy congestion of the lung.

The genital tract often showed involvement.   In the female there was congestion of the uterus with enlargement and accompanied sometimes by exudative plaques on the peritoneal surface and sometimes by the presence of a thin purulent exudate on cross-section. In the male changes were less frequent.   At times the testis showed congestion and appeared swollen and soft.   The seminal vesicles presented an opaque appearance with or without congestion.

As a routine procedure a culture was made from the spleen of all guinea pigs that came to autopsy.   In every instance where the postmortem findings indicated paratyphoid there was obtained from the spleen a microorganism which showed cultural and biochemical characteristics typical of the paratyphoid members of the Salmonella group.   Morphologically the microorganism was an actively motile, Gram-negative bacillus.   On agar it produced colonies which were transparent, flat, of a bluish cast, non-mucoid, with regular border, and reaching a size up to 4–5 mm. in diameter after 48 hours on a thinly seeded plate.   In broth a dense, even turbidity was produced in 24 hours with scant sediment and sometimes a partial surface film.   Hydrogen sulfide was produced from peptone, but no indole. Gelatin was not liquefied.   Of carbohydrates dextrose, maltose, mannitol, and dulcitol were fermented with acid and gas.   There was no fermentation with saccharose, lactose, and salicin.   In milk a transient acidity was produced with a slow change to an alkaline reaction.   After 10 days to 2 weeks the milk presented a yellowish, semitranslucent appearance.

The bacteriological technic employed at postmortem was as follows.

With young guinea pigs the entire spleen was removed with sterile forceps to an agar slant containing a small amount of bouillon at the base. With adult animals one or several pieces of spleen were torn off and added to agar. In special cases cultures were made from other organs and from the feces. The tubes were examined after 18 hours and 48 hours of incubation at 37°C. If growth had occurred microscopic examination was made for motility. *B. paratyphi* shows active motility after 18 hours incubation, whereas most of the other bacteria, such as strains of *B. coli*, encountered at times in the spleen are non-motile or very sluggishly motile after that period of growth. Non-motile bacteria were not further identified. If the microorganism was motile additional preliminary tests were made in some cases. Generally when an 18 hours motility was encountered a pure culture of *B. paratyphi* was obtained. In doubtful cases where the 18 hours growth showed a few motile forms additional tests were always made. A transfer from the original culture was made to malachite green-lead acetate bouillon. If the tube showed a brown precipitate with motile bacteria after 18 hours of incubation, a lead acetate plate was streaked and several of the brown colonies which developed transferred to agar slants. In some instances the fermentation reaction was tried in saccharose and lactose bouillon.

In all cases the cultures tentatively identified as *B. paratyphi* were tested with an agglutinating serum. The antiserums were obtained from rabbits immunized with a culture isolated from a typical case of paratyphoid early in the endemic period. The first serum employed showed an agglutination limit in a dilution of 1:25,600 for its homologous culture. With it were tested a representative number of cultures isolated during the active period of the epidemic and all cultures isolated during the endemic period through August, 1925. The second serum showed an agglutination limit in a dilution of 1:51,200 and was used for testing all cultures isolated from August, 1925 to the present time. The method employed in the direct agglutination tests was as follows. The growth on an 18 hour agar slant of the organism was removed with saline and the suspension diluted to a turbidity corresponding to 2.4 on the Gates scale. 0.5 cc. amounts of the antiserum, ranging by twofold dilution from 1:100 to 1:25,600, were pipetted into small tubes and 0.5 cc. of the fresh unheated suspension added to each, giving a final dilution range of 1:200 to 1:51,200. Appropriate controls were included. The reaction was read after 3 hours incubation at 37°C. and again after 18 hours at ice box temperature.

Of 110 cultures tentatively identified as *B. paratyphi* which were tested by one or the other of the two serums only one failed to agglutinate to the titer limit of the serum for its homologous strain. The single exception was a culture isolated from a sow which died early in the endemic period with typical paratyphoid lesions. The culture

showed positive presumptive tests for *B. paratyphi* but was agglutinated only through a dilution of 1:3,200 by a serum with titer limit in a dilution of 1:25,600. A number of cultures were encountered which showed spontaneous flocculation in saline. In some instances cultures showed slight agglutination in a dilution higher than that of the previously determined titer limit of the serum.

TABLE V.

Agglutination Limits with Guinea Pig Paratyphoid 922 and Mouse Paratyphoid I Antiserums.

Culture	Antiserum	
	Guinea Pig 922	Mouse I
Guinea pig paratyphoid 922	1:51,200	1:51,200
" " " IV	1:200	1:200
" " " VII	1:51,200	1:51,200
" " " VIII	1:51,200	1:51,200
" " " IX	1:51,200	1:51,200
Rabbit " 933	1:51,200	
Mouse " I	1:51,200	1:51,200
" " II	1:200	1:200
Rat " V	1:51,200	
Swine " IV	1:400	1:400
Calf " I	1:400	1:400
" " IV	1:400	1:400
B. choleræ suis X	1:100	1:100
" *enteritidis* Gaertner (Kral)	1:400	1:400
" " " (Mt. Sinai Hospital)	1:800	
" *paratyphi* a Schottmüller	1:200	1:200
" " b "	1:400	1:400
" " *aertrycke* 387	1:200	1:200
" *typhi* X	1:3,200	1:1,600

In general the agglutination tests showed a marked uniformity of the cultures isolated. A single strain of *B. paratyphi* thus appears to have been the causal organism of the epidemic during both the active and the latent stages. The one culture which showed an outstanding difference in agglutinability may have been a different type of *B. paratyphi*. If so, it was apparently of no further significance as low agglutinating cultures were not subsequently encountered.

The relationship of the causal organism of the epidemic to other

members of the Salmonella group was studied by direct agglutination. The agglutination limits of 18 members of the Salmonella group and of one strain of *B. typhi* with an antiserum prepared against the epidemic strain are given in Table V. There are included, in addition, the agglutination limits with an antiserum prepared against a culture of Mouse Typhus I. This culture was originally isolated by Lynch (9) during a spontaneous epidemic of paratyphoid in mice. The two mouse strains were obtained from The Rockefeller Institute through Dr. Ida W. Pritchett. The *aertrycke* strain was obtained from Dr. E. O. Jordan. The rabbit strain was isolated from the stock of this Department in 1925. Of the guinea pig strains,

TABLE VI.

Agglutinin Absorption Tests with Guinea Pig and Mouse Typhus I Serums.

Serum	Culture	Direct agglutination	Absorbing culture	Absorbing dose	Agglutination after absorption	Culture
G.P.T. 922	G.P.T. 922	1:51,200	G.P.T. 922	1:5	1:50	G.P.T. 922
			" 922	1:5	1:50	M.T. I
	M.T. I	1:51,200	M.T. I	1:5	1:100	G.P.T. 922
			" 1	1:5	1:100	M.T. I
M.T. I	" I	1:51,200	" I	1:5	1:50	" I
			" I	1·5	1:50	G.P.T. 922
	G.P.T. 922	1·51,200	G.P.T. 922	1:5	1:100	M.T. I
			" 922	1:5	1:100	G.P.T. 922

Culture 922 was isolated during the present epidemic. Culture IV was isolated by Dr. P. A. Lewis from a Boston epidemic in 1908. Culture VII was isolated by Dr. Theobald Smith in 1913, Culture VIII by Dr. Carl TenBroeck in 1914 from The Rockefeller Institute stock, and Culture IX from the same stock in 1917. The other cultures were selected from the collection maintained at the Department.

Reciprocal absorption tests, as a further indication of the relationship of the various cultures to the epidemic strain, were made only with Mouse Typhus I. The absorptive capacity of the mouse strain was compared with that of the guinea pig strain, Culture 922. The

method suggested by Krumwiede and his associates (10) was employed.   The absorptive capacities of the two cultures in homologous and heterologous serums are given in Table VI.

<div align="center">DISCUSSION.</div>

There was no indication that the active stage of the epidemic was preceded by a pre-epidemic period marked by occasional deaths. Routine examination of all guinea pigs that died among the stock had been made prior to the appearance of the early cases and nothing suggestive of the disease had been encountered.   The infection began abruptly, ran a moderately acute course for a period of 8 weeks, and then subsided in part.   The active stage was followed by a long protracted endemic period characterized by sporadic deaths.   During both stages female guinea pigs showed a somewhat greater susceptibility to the infection than did the males.   This difference in susceptibility was well brought out with the breeders during the active stage.   Similar differences in susceptibility have been noted with other outbreaks of paratyphoid.   With the adult animals, breeders, and older stock guinea pigs, the disease did not run a particularly acute course.   At postmortem it was customary to find well developed lesions the nature of which would indicate that the disease had been in progress for about 1 to 2 weeks.   With the younger guinea pigs, unweaned and recently weaned individuals, the disease was more acute.   The unweaned animals sometimes died 2 to 3 days after birth with no noticeable manifestations save a slightly enlarged and congested spleen yielding B. paratyphi in pure culture.   The cultures isolated at postmortem, with one exception, were of the same strain of B. paratyphi.

The comparative study of the agglutinative affinities of the epidemic strain was not extensive enough to permit definite conclusions as to its position in the Salmonella group.   Direct agglutination indicated that the epidemic strain was closely related to three stock strains of guinea pig origin and to three strains from other rodents.   It showed only remote relationship to a fourth guinea pig strain, to several types of human B. paratyphi, to B. enteritidis Gaertner, and to a group of strains isolated from domestic animals.   Absorption tests with Mouse Paratyphoid I and with Guinea Pig Paratyphoid 922 gave

further proof of a close relationship between the two strains, if not of their actual identity. The indicated relationships are in harmony with the early work of Wherry (3) and the more recent work of Krumwiede, Valentine, and Kohn (11). The latter investigators studied the agglutinative affinities of fifteen strains from guinea pigs. Thirteen of the strains were alike and identical, as shown by agglutinin absorption, with two strains from mice and one each from a rabbit and a cat. They concluded that a distinct paratyphoid type or group of bacilli was encountered in spontaneous infections of laboratory animals especially rodents.

It is apparent that other members of the Salmonella group may be encountered, at times, in natural infections of the guinea pig and of other small animals as well, strains closely related to *B. enteritidis* Gaertner and but remotely related to the type discussed. Likewise, it is to be expected that the rodent strain will be found occasionally in larger animals exposed to infection through contact with rats and mice.

SUMMARY.

The course of a natural outbreak of paratyphoid in a normal guinea pig population is outlined. A description is given of the gross pathology of the disease. The bacteriology of the causal organism is discussed with particular reference to its agglutinative affinities.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

II. Factors Involved in the Transition from Epidemic to Endemic Phase.

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The active stage of the epidemic of guinea pig paratyphoid (1), occurring in the summer months of 1924, was followed by a long protracted endemic period studied up to June, 1926. During this period the percentage mortality showed considerable fluctuation from month to month. It was low when compared with the rate during the active stage except during the hot months of 1925 when there was a slight increase in the fatal cases. In the hope that a study of all cases of paratyphoid occurring subsequent to the early epidemic period might offer some explanation of the drop from the epidemic to the low endemic stage, attention was focused in particular upon the dams and their litters.

It has already been stated that during the epidemic period the mortality among the sows was high. During the later period and up to the present, June, 1926, only 4 sows died of the disease. During the endemic period all sows whose young showed infection with *B. paratyphi* were taken from the breeding house with the surviving young and placed together in a large screened box for observation. These sows and the remainder of the litter were killed at intervals and cultures made from spleen, uterus, gall bladder, and cecum. The blood serum was tested for specific agglutinins towards the epidemic strain. In all, 35 sows with at least one fatal case of paratyphoid in each litter were segregated during the period of observation.

On the basis of the postmortem findings the sows could be divided into three groups. The first group comprised the fatal cases. Death

TABLE I.

Bacteriological Data of Isolated Guinea Pigs and of Their Unweaned Young Which Had Succumbed to Paratyphoid Infection.

No. of guinea pig	Age at death in mos. k. = killed d. = died	Data of sow*						Data of young				
		Spleen	Uterus	Cecum	Gall bladder	Fetal spleen	Serum titer	No. in litter	Deaths	Date of death	Age in days	Spleen culture
										1924		
1	30 (k. Mar. 3, 1925)	+					1:320	3	1	Oct. 16	21	+
2	11 (k. Mar. 25, 1925)	—	—	—	—		1:160	3	1	" 24	6	+
3	25 (k. Mar. 25, 1925)	+	—	—	—		1:80	3	1	" 27	10	+
4	28 (k. Apr. 7, 1925)	—	+	—	—		1:160	4	1	Nov. 1	5	
										1925		
5	12 (k. Apr. 22, 1925)	—	—**	—	—	—	1:320	4	1	Jan. 24	7	+
6	10 (k. Mar. 19, 1925)	—	—**	—	—	1.+ 2.— 3.—	1:80	4	1	Apr. 13	3	+
7	11 (k. Apr. 18, 1925)	—	—	—	—		1:80	4	1	" 13	3	+
8	15 (k. Apr. 21, 1925)	—	—	—	—		<1:10	4	1	" 16	3	+
9	25 (k. Apr. 18, 1925)	+	—	—	—		1:40	4	3	" 16 " 16 " 16	8 8 8	+ + +
10	8 (d. Apr. 20, 1925)	+	+	+	+			5	3	Apr. 18 " 18 " 20		+ + +
11	27 (k. Apr. 29, 1925)	—	—	—	—		1:160	2	1	" 25	10	+
12	12 (d. June 13, 1925)	+	+					4	2	June 12 " 12	8 8	+ +
13	16 (k. Oct. 2, 1925)	—	—	—	—		1:40	3	2	Sept. 17 " 17	12 12	+ +
14	17 (k. Oct. 2, 1925)	—	—	—	—		1:320	3	1	" 18	8	+
15	17 (k. Oct. 2, 1925)	—	—	—	—		1:20	4	1	" 19	11	+
16	16 (k. Oct. 13, 1925)	+	—**	—	—	1.— 2.—	1:80	4	3	" 29 Oct. 8 " 12	21 30 34	+ + +

* + = positive cultures; — = negative cultures.
** Culture included placenta.

TABLE I—*Concluded.*

No. of guinea pig	Age at death in mos. k. = killed d. = died	Data of sow*						Data of young				
		Spleen	Uterus	Cecum	Gall bladder	Fetal spleen	Serum titer	No. in litter	Deaths	Date of death	Age in days	Spleen culture
17	7 (k. Oct. 19, 1925)	−	−	−	−		1:1,280	2	2	*1925* Oct. 5 / " 5	11 / 11	+ / +
18	21 (d. Mar. 1, 1926)	+	+	+	+			5	4	*1926* Feb. 19 / " 19 / " 23 / " 23	3 / 3 / 7 / 7	+ / + / + / +
19	20 (d. Feb. 27, 1926)	+	+	+	+			5	4	" 23 / " 25 / " 25 / " 25	7 / 9 / 9 / 10	+ / + / + / +
20	32 (k. Mar. 8, 1926)	−	−	−	−		1:320	3	1	" 16	6	+

occurred some time after parturition. The sows all showed typical focal lesions in the spleen together with other manifestations of paratyphoid. *B. paratyphi* was obtained in cultures from the spleen and feces, and in two of the cases from the uterus and gall bladder. The blood serum, tested for agglutinins in only one instance, showed no agglutination in a dilution of 1:10. With these cases the majority of the young in the litter died.

The second group included the majority of surviving cases, *i.e.* sows which had recovered from a slight attack of paratyphoid or had been carriers with localization of *B. paratyphi* in the spleen. Within the litters only part of the young succumbed to paratyphoid. The sows of this group showed serum agglutinins for the epidemic strain of *B. paratyphi* in dilutions ranging from 1:20 to 1:1,280. The organism was recovered from the spleen in only two instances. Cultures from the feces and gall bladder were uniformly negative. In one case *B. paratyphi* was obtained from the uterus and in one case from a fetal spleen. It sometimes happened that the sow had been

rebred before removal for the nursing period. The spleen and liver sometimes showed a few foci, with a negative culture, together with other indications of past infection.

The sows of the third group showed no specific agglutinins in a dilution of 1:5. Cultures from the spleen, gall bladder, uterus, and feces were uniformly negative. The spleen and other organs did not show gross changes suggestive of former disease. The sows of the group were regarded as sometime fecal carriers of the organism. The postmortem findings of a selected number of the isolated sows, together with the young of their litters which succumbed to paratyphoid, are given in Table I.

This material affords a basis for certain inferences. (1) The dams, as might have been anticipated, were far more resistant than their offspring. The survivors appeared normal when killed later. (2) In those animals that died, the gross lesions appeared equal in extent and severity to those encountered in the epidemic period. (3) Intrauterine transmission of the infecting agent to the fetus was demonstrated in one case. (4) The bacilli were excreted either by way of the digestive tract, the uterus, or the mammary gland, which in one instance contained a number of abscesses. *B. paratyphi* was regularly isolated from the feces of those sows which succumbed to active infection shortly after parturition. With the sows which showed evidence of inactive infection or of recovery from a past active infection the causal organism was rarely recovered from the feces except when examination was made immediately after segregation. It seemed probable, however, that the majority of the sows were excreting the organism at the time of parturition. If such was the case, it would appear that the carrier state, as evidenced by the presence of *B. paratyphi* in the feces, was of relatively short duration.

To obtain indirect evidence bearing on the persistence of the causal organism in the feces a small series of guinea pigs was fed *B. paratyphi* and the feces subsequently cultured for the organism. The series included one lot of two older stock guinea pigs and one lot of four recently weaned pigs. For comparison a series of eight mice was included. Cultures made from feces collected before feeding were all negative. The animals were fed by pipette on 2 successive days. The guinea pigs received a total volume of 1 cc. of an 18 hour broth

culture of *B. paratyphi*, approximately 800,000,000 organisms. The mice received a total of 0.1 cc. of the same culture, or 80,000,000 organisms. Cultures were made 4 days after the last feeding and then every 7 days until negative. The mice invariably showed a long continued carrier state and were killed before the excretion of the organism had terminated.

The method used in culturing was as follows:

A heavy suspension of fresh feces was made in plain bouillon and incubated at 37°C. for 3 to 5 hours. A small amount of the culture, 0.2 cc., was pipetted into a tube of fermented bouillon containing lead acetate (0.25 cc. of a 1 per cent solution in 5 cc. of medium) and malachite green (0.1 cc. of a 0.1 per cent solution in 5 cc. of medium), and incubated overnight. In the presence of a deep brown or black precipitate and motility, or either, a drop was streaked on the surface of a lead acetate-fermented bouillon agar plate and a thin layer of the same medium superimposed. Deep brown colonies, after 18 hours of incubation, were fished to agar slants and finally tested with a specific antiserum. In the absence of *B. paratyphi*, the lead acetate-malachite green broth generally showed a very scant turbidity, no change in color of the precipitate, and no motility. In the presence of *B. paratyphi* there was always motility, a moderate turbidity, and a browning or blackening of the precipitate.

The guinea pigs were killed shortly after the indicated termination of the fecal carrier state. The mice were killed after a prolonged period of continued excretion. The results of the feeding experiments with the two lots of guinea pigs and with the mice are given in Tables II and III.

Per os administration of *B. paratyphi* to these guinea pigs was not followed by active disease. With the older stock pigs there was an immediate excretion of the organism which continued for a period of approximately 3 weeks. The feces subsequently became negative. The guinea pigs were then killed and autopsied. Lesions were not observed. The organism was recovered from a piece of Peyer's patch of the small intestine in one case and from the spleen in one case. Agglutination tests gave evidence of an immunity response on the part of the host. With the recently weaned guinea pigs there was a delayed and subsequently irregular excretion of *B. paratyphi*. One guinea pig gave but a single culture of the organism from the feces. The fecal excretion of *B. paratyphi* terminated sometime

TABLE II.

Results of Fecal and Postmortem Cultures from Guinea Pigs Fed B. paratyphi.

No. of days after last feeding	Stock guinea pigs		Recently weaned guinea pigs			
	1	2	3	4	5	6
4	Feces +	Feces +	Feces −	Feces −	Feces −	Feces −
11	" +	" +	" +	" −	" +	" +
18	" +	" +	" −	" −	" +	" +
25	" −	" −	" +	" +	" −	" −
27	" −	" −	" +			
28	Killed Spleen + Peyer's patch*** + Serum agglutinins 1:320	Killed Spleen − Peyer's patch + Serum agglutinins 1:640				
32			" −	" −	" −	" −
34			" −	" −	" −	" −
35			Killed Spleen + Peyer's patch + Serum agglutinins 1:40	Killed Spleen + Peyer's patch − Serum agglutinins 1:80	Killed Spleen − Peyer's patch − Serum agglutinins 1:80	Killed Spleen + Peyer's patch − Serum agglutinins 1:20

*** "Peyer's patch" means that a piece of intestinal wall containing a Peyer's patch was cut out and transferred with some fecal material to an agar slant.

TABLE III.

Results of Fecal and Postmortem Cultures from Mice Fed B. paratyphi.

No. of days after last feeding	M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8
4	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +
11	" +	Died on 10th day Spleen + Feces +	" +	" +	" +	" +	Died on 7th day Spleen + Feces +	" +
18	" +		" +	" +	" +	" +		" +
25	" +		" +	" +	" +	" +		" +
32	" −		" +	" +	" +	" +		" +
39	" +		" +	" +	" +	" +		" +
46	" +		" +	" +	" +	" +		" +
53	" +		" +	" +	Killed on 49th day Spleen + Feces +	Killed on 49th day Spleen + Feces +		Killed on 49th day Spleen + Feces +
56	Killed Spleen + Feces +		Killed Spleen + Feces +	Killed Spleen + Feces +				

between the 3rd and 4th week. At autopsy the organism was recovered from the intestine in one case and from the spleen in three out of four cases. The immunity response was weak as compared with that of the older guinea pigs.

With mice the feeding of the organism resulted in death in two cases. The survivors showed a persistent fecal excretion of *B. paratyphi*. The animals were killed at the end of the 7th or 8th week after the last feeding and in every case a positive culture was obtained from the spleen and from the feces.

During the endemic period, guinea pigs in other lines of research were scrutinized as far as this was possible A series of 32 stock guinea pigs had each received intraperitoneal injection of material containing *B. abortus*. At varying intervals after injection the animals were killed and the entire spleen cultured. In two cases pure cultures of the epidemic strain of *B. paratyphi* were obtained from the spleen. No gross manifestations of infection were noted in either case. Another series of 10 guinea pigs had received intraperitoneally sublethal doses of a virulent strain of *B. coli*. The animals were killed on the 5th day and the entire spleen cultured in every case. A mixed growth of the injected organism and the epidemic strain of *B. paratyphi* was obtained from one animal. The spleen showed only the exudative membrane occasioned by the *B. coli* strain. *B. paratyphi* was not recovered from the feces.

Several series of stock guinea pigs, selected at random from the benches, were examined during the endemic period for the fecal carriage of *B. paratyphi*. Of 40 animals cultured the organism was recovered from the feces in only one. This guinea pig was one of a series of 12 removed from a bench shortly after the appearance of a fatal case of paratyphoid in it. The fecal culture was positive immediately after segregation. Cultures made 6 and 9 days later were negative. The animal was killed on the 21st day. *B. paratyphi* was obtained in cultures from the spleen but not in cultures from the feces or in cultures inoculated with a piece of a Peyer's patch.

A survey of the possible agencies transmitting the virus from cage to cage and group to group focused attention upon a few gray mice which gain access from time to time to the building from the fields and woods around it. Data already presented clearly showed that

. white mice freely discharge the guinea pig strain for some time after having been fed. Early experience of one of the authors with wild mice indicated no appreciable difference of gray and white mice towards hog cholera bacilli. The agency of wild mice was furthermore suggested by the occasional occurrence of a paratyphoid disease among breeding rabbits kept in another wing of the same building. The cases were rare, not more than several in the fall of 1924, a few in 1925, and one in April, 1926. The lesions were characteristic of the less acute type of paratyphoid infection in rabbits, such as necrotic follicles in appendix, necrotic follicles in Peyer's patches, enlarged, firm, congested spleen, and, very rarely, necrotic foci in liver. The isolated bacilli agreed culturally and serologically with the guinea pig strain. Several wild mice examined recently did not carry *B. paratyphi* however.

<center>DISCUSSION.</center>

Spontaneous epidemics among small animals have not been studied in the past with the care warranted by the importance of the phenomenon. Most investigators have perhaps reached the conclusion that the many unknown factors in spontaneous epidemics stand in the way of any trustworthy conclusions to be derived from such study. Hence the experimentally controlled epidemiological investigations of recent years (Topley (4), Amoss (3), Webster (5)). Nevertheless it is the natural epidemics from which must be drawn the problems to be solved. Without such study the problems do not readily present themselves or they may be overshadowed by the artificial conditions of the experiment. In these pages by the natural epidemic is meant the occurrence of disease without the use of cultures artificially administered. In the epidemic reported above the question as to the decline of the epidemic to the endemic level was uppermost. Among the hypotheses to present themselves the following appear to us as involving the major factors.

(a) The sudden, acute, highly fatal onset of the infection may be interpreted as an attack upon a number of animals below the normal resisting power of the species which had accumulated during the 6 years of freedom from disease. This accumulation may be due to fortuitous sexual selection going on in the population or to spon-

taneous variation in natural resistance. We may assume that most. of the individuals belonging to this category were wiped out in the early weeks of the disease when the breeders were the chief victims. This theory is supported by the fact that no epidemic rise has occurred since the fall of 1924. The occasional appearance of an animal with marked lesions may be due to individually depressing environmental causes, to which the group is subject, as well as to a reappearing, less resistant stock.

(b) Another hypothesis assumes an increase in specific immunity towards B. paratyphi following the ingestion of minute doses which caused the epidemic to subside towards the endemic level. This theory is not well supported. There should have been found a less abrupt drop in mortality and many more carriers in the exposed guinea pigs during the endemic period. It is not to be denied, however, that the resistance of certain groups may have been raised by the ingestion of minute doses in the early epidemic phase.

(c) A third hypothesis assumes a decline in the virulence of the bacillus in passages through successive guinea pigs. Stated differently, this hypothesis assumes that the virus was introduced from a somewhat higher level of virulence, possibly from some other host species, and after several passages was thereby brought into equilibrium with the new host. This hypothesis was tested in several ways. A culture from one of the earliest acute cases was at hand to be tested comparatively with a culture from the later endemic period. The former had been isolated July 14, 1924; the latter March 19, 1925. The possible unreliability of any comparative tests was kept in mind in view of the additional 8 months of artificial cultivation of the early strain. Subcutaneous and intraperitoneal injection of graded dilutions prepared from 18 hour bouillon cultures of the two strains was made in guinea pigs of approximately 350 gm. weight. With the series which received subcutaneous injection a slight difference in virulence between the two cultures was encountered. The older culture showed a virulence approximately four times that of the endemic culture. A dilution range of the bouillon culture of 1:20 to 1:160 was employed. Within that range extensive ulceration at the site of injection resulted with both cultures. Death occurred in approximately 10 days with the guinea pigs which

had received the older culture in a dilution of 1:80. Of the guinea pigs which had received the endemic culture death occurred only following a dilution of 1:20 after a slightly longer period.

The results obtained from the series which had received intraperitoneal injection of the two cultures were less suggestive of a difference in virulence. The intraperitoneal injection of the older culture in a dilution of 1:500 resulted in death in 48 hours. With the endemic culture death did not occur until 8 days after the injection. Dilutions higher than 1:1,000 produced death in 10 to 14 days or more with advanced focal lesions in the spleen and liver. In several later series which had received graded dilutions intraperitoneally no consistent difference in the lethal dose was observed. The guinea pigs employed in the tests were descendents of the original infected population several generations removed from the active period of the epidemic. Individual differences in natural susceptibility might be sufficient to account for fluctuations in the response to graded dilutions.

A second method of comparing the virulence of the bacillus in the epidemic and the endemic period was to study the lesions of cases occurring in these periods. Tissues from 31 animals were examined in fixed and stained sections. This material consisted of breeders, very young and young adult animals, and of cases from the early epidemic and the late endemic period. The material was from both naturally dead and chloroformed animals. A comparative study of this miscellaneous material did not bring to light any histological characters clearly distinguishing the early and subsequent periods. The various changes in spleen, liver, lymph nodes, and intestines were found at all periods and in various ages and a detailed description of these changes is therefore omitted.

A change in virulence has thus not been demonstrated. It must be granted, however, that the methods employed to show such transformation are relatively crude. The change from an epidemic to an endemic level is obviously due to a number of cooperating and mutually interfering factors and all we can state at present is that it actually occurs in all epidemics sooner or later in a stationary population, in spite of the fact that such groups of bacteria as streptococci, pneumococci, and bipolar organisms (hemorrhagic septicemia)

may be raised in virulence by passages through a series of animals. The increase in virulence of the paratyphoid group by passages is not clearly proved by experiment. Moore (2) failed to increase the virulence of hog cholera bacilli by passages through rabbits. Webster (5) failed to show any rise in virulence. Lockhart (6) concludes that the virulence of a single strain of *B. aertrycke* may be significantly increased as the result of animal passage, but in the text he admits that such increase cannot be brought about with any regularity. A prolonged experience of one of us with the hog cholera bacillus since 1885 leads to the conclusion that passages tend downwards rather than upwards in capacity to kill. If experiments in epidemiology are to elucidate natural phenomena, it is to be borne in mind that passages through a series of susceptible animals by inoculation do not imitate nature, for in the spontaneous disease the infectious agent is transmitted through the digestive tract and of what goes on there nothing is known. That the selective action of the host is the same through enteral and parenteral channels may at least be doubted. Furthermore, virulence is regarded by some as the capacity to multiply and kill, whereas in passages the change may be towards greater tenacity of life in the tissues rather than the capacity to multiply and thus destroy. The infection in the population under consideration has now been followed through three summers, the first being that of the original epidemic period. In 1925, the year following the initial outbreak, the mortality was highest during the summer months, reaching a peak in June, with a rate of 4.1 per cent, followed by a decline in July. For 1926, up to September, the mortality was likewise greatest during the summer with a high rate of 6.4 per cent in August. A rough evaluation of the three summer periods may be made by comparing the combined rates for July and August of each year. It was during that period that the majority of deaths occurred at the time of the original epidemic. Taking into consideration the few deaths that occurred in September, 1924, which were included in the original rate computed for that period, the percentage mortality for July and August, 1924, was 16.7 per cent. The rates for a corresponding period of time in 1925 and 1926 were 3.7 per cent and 6.6 per cent, respectively, definitely lower than the rate of the epidemic period.

The infection has therefore been followed long enough to eliminate the various secondary influences such as changes in the character of the food during the four seasons and changes in humidity and temperature. During the occasional excessive heat periods of the summer months deaths among adult guinea pigs have occurred not referable to anything but the heat.

SUMMARY.

Factors bearing on the maintenance of paratyphoid in an endemic state are discussed. There was no evidence of any increase nor any · clearly demonstrable proof of a decline in virulence of the causative organism. This persisted within the breeding stock and it is suggested that the sows constituted the chief focus for dissemination of the organism to their young and from these to the population at large. Evidence is presented that the carriage of *B. paratyphi* in the feces was of relatively short duration. Fecal carriage of *B. paratyphi* was commonly associated with a localization of the organism in the spleen. Since it is obvious that some factor or factors must have changed in the transition from epidemic to endemic phase in the presence of younger generations, the hypothesis is tentatively presented that the transition from epidemic to endemic phase is due to a combination of the weeding out of individuals of low natural resistance with a gradual adjustment of the invading organism to the population on a lowered level of virulence.

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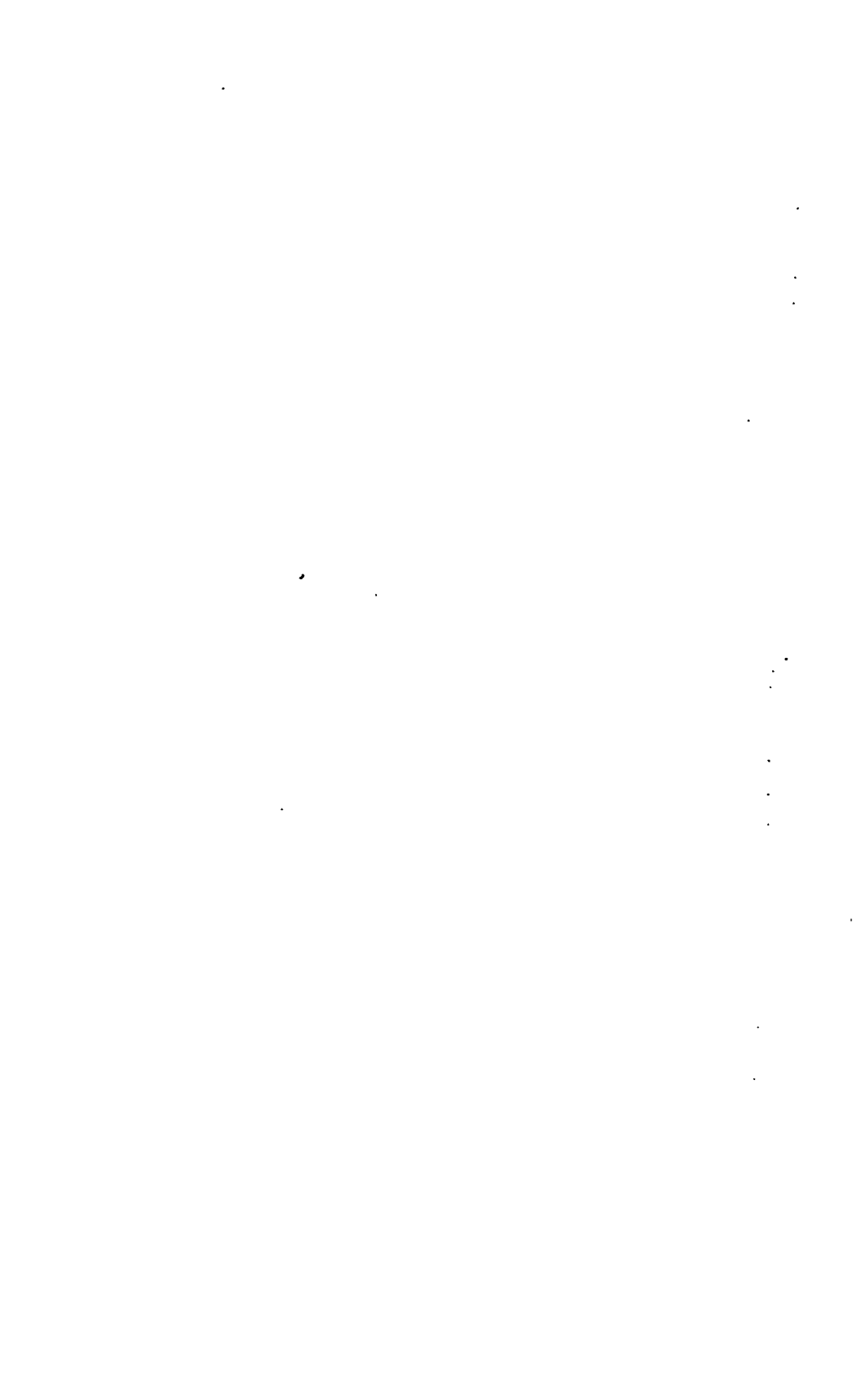
THE BIOLOGICAL CHARACTERS OF A MUCOID VARIANT OF BACILLUS PARATYPHI FROM GUINEA PIGS.

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A mucoid type of *B. paratyphi* was encountered during the course of a spontaneous epidemic of paratyphoid in a previously normal guinea pig population. The active stage of the epidemic occupied a period of 8 weeks in the summer of 1924 and was followed by an endemic stage which has continued with sporadic deaths for 2 years. The normal, smooth type of organism was isolated from the majority of guinea pigs that succumbed to the infection during both stages. Early in the endemic stage, however, two cultures were obtained which differed from the normal in that they were distinctly mucoid and flocculated spontaneously in bouillon. The first culture, No. 1004, was isolated from the spleen of an unweaned, female guinea pig 13 days old. At autopsy the spleen was enlarged, congested, and covered with foci. A mixed growth of the smooth and mucoid types was obtained from the spleen upon plating from the original tissue culture. The second culture, No. 950, was likewise recovered from an unweaned female. The guinea pig died 6 days after birth. At autopsy a slight congestion of the lung was encountered but no typical gross lesions of paratyphoid. A pure growth of the mucoid type was obtained from the spleen.

Mucoid types of the human strains of *B. paratyphi* have been encountered and reported in the literature. Fletcher (1) isolated mucoid varieties of *B. paratyphosus* b from the feces of two chronic carriers and of *B. aertrycke* from the feces during an acute case of meat poisoning. The variants were motile, often small, coccoid bacilli which produced a mucoid substance apparently not as a true capsule and formed large, wet colonies on agar. They were agglutinated in low dilution by an antiserum specific for the normal type. One of the *B. paratyphosus* b variants did, however, absorb the agglutinins from the serum. The other did so imperfectly.

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There was no absorption by the *B. aertryckc* variant from an *"aertryckc"* serum. After several daily transplants in peptone water followed by plating the mucoid varieties gave rise to varying numbers of normal colonies. The normal type could likewise be recovered from agar slants of the variant which had been held for several days. The mucoid type was never recovered from the normal, non-mucoid culture.

Thjøtta and Eide (2) isolated a mucoid variant from the urine of a chronic paratyphoid patient. The organism was small, showed few motile forms, and produced a mucoid intercellular substance. It formed large, rounded, wet colonies on agar. Its agglutinability and absorptive capacity were judged to be comparable with those of the normal type, except that the reactions were slower. The mucoid character was regarded as constant although serial plating from single colonies showed at first the production of a small number of normal colonies. The variant was slightly less virulent for mice, upon injection, but the difference was not held to be significant.

Walker (3) found that the prolonged cultivation of *B. paratyphosus* b in bouillon containing specific antiserum gave rise to the production of a mucoid type of the organism. The variant was almost completely non-motile and formed large, umbilicated, moist, waxy colonies on agar. It was agglutinated in low dilution only by *B. paratyphosus* b antiserum. An old agar culture of the variant was transplanted daily in bouillon and reverted to the normal type losing its mucoid nature and becoming motile and agglutinable.

Krumwiede, Cooper, and Provost (4) encountered mucoid variants of *B. paratyphosus* b in the feces of paratyphoid carriers. The mucoid type showed some tendency to produce smooth and rough varieties on cultivation. Absorption tests with the mucoid variant gave anomalous results. It showed little ability to absorb the agglutinins from "rough" and "smooth" antiserums. The low agglutinability and absorptive capacity indicated that the mucoid type differed antigenically from the rough and smooth types of the same strain. It was not believed that the antigenic difference was due solely to the mucoid characteristic.

In Germany considerable attention has been directed towards the appearance of so called *Schleimwall* colonies in cultures as a means of differentiation between *B. paratyphi* b Schottmüller and the meat-poisoning types, *B. enteritidis* Gaertner and Breslau. Freshly isolated cultures of the former when kept at room temperature after 24 hours of incubation at 37°C. were regularly found to form colonies which showed an outer zone or wall radially striated and slimy in nature due to the development of an intercellular material. Such colonies were supposed to be imperfectly developed with the Gaertner strains and absent with the Breslau strains A critical review of the phenomenon together with a discussion of the rôle of chemical stimuli, such as sodium chloride and increased H ion concentration, in initiating the growth is given by Elkeles (5, 6).

The two mucoid variants under discussion were short, rather plump bacilli. With ordinary methods of staining and in wet preparations

they showed little deviation in size from the smooth type. Sometimes the latter appeared a trifle longer, more distinctly rod-shaped, while the mucoid types more closely approached a coccoid shape. Films made from an 18 hour agar growth fixed with acetic acid and stained with carbolfuchsin showed a faintly stained envelope of indefinite outline surrounding individual cells or pairs of cells. With heavier films the bacteria appeared embedded in a matrix of similarly stained material. With definitely encapsulated bacteria the acetic acid-fuchsin method showed an envelope with distinct boundary. As noted by other workers, the surrounding envelope displayed by the mucoid variants was more in the nature of a viscid intercellular substance than a true capsule. The same method applied to films made from the smooth type showed no enveloping material. Whatever the nature of the envelope it did not appear to affect materially the motility of the organisms. 6 hour bouillon cultures of the mucoid variants showed fine clumps of bacteria together with many free forms. The latter were very actively motile. The clumps also displayed considerable motility. With continued incubation the clumps increased in size and tended to become fixed. Small numbers of free forms were always observed, through 36 hours growth, and these retained their motility.

The mucoid variants gave biochemical reactions identical with those of a smooth type of the epidemic strain. The three cultures fermented dextrose, maltose, levulose, galactose, dulcitol, arabinose, mannitol, and xylose with the production of both acid and gas. Neither acid nor gas was produced from saccharose, lactose, and salicin. Hydrogen sulfide was formed in peptone media but no indole. Gelatin was not liquefied. A strong terminal alkalinity was produced in milk attended by a partial clearing of the medium which presented a slightly yellowish, semitranslucent appearance after 10 days.

The mucoid types differed markedly from the smooth type in the character of growth in bouillon and on agar. The smooth type gave rise to a heavy, even turbidity in bouillon with little tendency towards settling except upon prolonged cultivation. Sometimes a slight scum formed on the surface of the medium but a definite, complete film was never observed. With the mucoid types a spontaneous flocculation followed by sedimentation occurred in bouillon. A 48

hour culture showed a moderate to heavy sediment, a slightly turbid supernatant with floccules in suspension, and generally a distinct surface film.

On agar the smooth type formed transparent, flat, moist but non-mucoid colonies which had a bluish cast and regular border. The colonies reached a size of 4–5 mm. after several days on a thinly seeded plate and tended to become slightly convex showing a deeper colored central zone which gave them a ringed appearance. The mucoid types both formed slightly larger colonies which reached a size of 5–6 mm. after 4 days at room temperature. Sometimes the growth was stringy but more often waxy in nature forming heaped up masses when touched with a needle. The colonies usually became differentiated into a central zone which was either wrinkled with radial striations or granular, and a smooth, outer rim of varying width. At times part or all of the colonies showed only a raised, smooth, central zone. Such colonies when picked into bouillon and plated gave rise to the wrinkled and granular type as frequently as they did to the undifferentiated type of colony.

On agar slants the mucoid types produced a heavy growth which was stringy in nature. On older slants the growth became wrinkled and less definitely viscid. Saline suspensions were formed with difficulty and with the usual salt concentration spontaneous flocculation always resulted. In distilled water and in solutions of reduced salt concentration, 0.4 per cent and less, the suspensions were more stable and showed only a scant sediment upon standing.

The experience of other workers with mucoid variants of the human strains of *B. paratyphi* indicated a tendency towards reversion to the normal type upon culturing. Likewise there appeared to be a tendency for the normal strains, in old culture or under special conditions of culture, to split off mucoid types in varying numbers. Inasmuch as one of the present guinea pig variants was isolated together with the normal type of *B. paratyphi*, it was regarded of interest to determine whether a similar reversion and splitting occurred with the animal strain. The presence of the variant in the guinea pig host might be explained by such a splitting from the normal type of organism.

The two mucoid types were plated on agar from 18 hour bouillon

cultures, a single colony of each type picked into bouillon and again plated after 18 hours. The procedure was repeated through 12 generations, 6 in bouillon and 6 on agar. The bouillon tubes always showed the characteristic growth with sediment, spontaneous flocculation, and surface film. On agar there were produced only waxy or viscid colonies with or without differentiated zones. Daily bouillon cultures of the two types were made through 30 generations and plated at intervals. The results were the same. The characteristic growth was maintained in bouillon and on agar.

The intraperitoneal injection of the mucoid types in low dilution into guinea pigs resulted in death and the recovery of the organism from the visceral organs and the peritoneal exudate. The growth was typically mucoid on agar slants and transplants in broth yielded the usual flocculating growth which on plating produced only waxy or viscid colonies. Similar results were obtained with cultures isolated from rabbits and from mice which had received intraperitoneally fatal doses of the organism.

There was no indication that repeated culturing of the mucoid variants in ordinary media caused a reversion to the normal type. Nor did there appear to be any reversion attendant upon the growth of the variants under parasitic conditions in experimental animals.

Six normal strains of *B. paratyphi*, isolated from guinea pigs which had succumbed to paratyphoid, were similarly carried through 8 successive generations on media, 4 in broth and 4 on agar. The bouillon tubes invariably showed an even turbidity with no flocculation and no surface film. On agar the colonies were all of the same smooth, non-mucoid type. A single strain was carried through 30 generations in bouillon with intermittent plating for colony characteristics. A change occurred in the nature of the growth in bouillon during the series of transfers. The culture developed a surface film and showed a slight tendency towards flocculation with increased sediment. On agar, however, there was no change noted in the nature of the colonies. They were always of the smooth, non-mucoid type.

The same culture was carried through 3 generations in bouillon containing 10 per cent specific antiserum and in 5 per cent peptone water. The transfers were made every 4th day. Plates were poured

from the two kinds of media on the 4th day after the third transfer. In both instances the plates showed two types of colonies in approximately equal numbers, the typical smooth colony and a more opaque type with an irregular, waxy border. None of the colonies were mucoid. The first type produced the usual even turbidity in bouillon. The second type showed spontaneous flocculation in bouillon with sediment but no surface film.

On several occasions cultures were obtained from the spleens of guinea pigs which had received intraperitoneally non-fatal doses of the smooth type of *B. paratyphi*. The spleen, liver, and lymphoid tissue of the intestinal tract gave evidence grossly of some structural change. The blood serum showed a moderate titer of specific agglutinins. The cultures on plating yielded only the smooth type of colony.

There was no indication that the normal, smooth type of *B. paratyphi*, of the present epidemic strain, yielded mucoid variants either with prolonged cultivation in ordinary media or in special media designed to stimulate variation. Growth in the latter media did, however, give rise to a rough type of variant. Moreover the smooth type failed to show any tendency towards variation in the guinea pig under conditions favorable for variation, that is in animals which had acquired some degree of resistance.

The relationship of the two mucoid types to the smooth or normal type of *B. paratyphi* was studied with the aid of the agglutination reaction. Antiserums were prepared by intraperitoneal injection of the three cultures into rabbits. Serums of high titer were obtained. The tests were made in approximately 0.4 per cent salt solution in order to reduce spontaneous flocculation. Readings were made only after 3 hours of incubation at 37°C. With continued incubation a scant flocculation occurred in the controls of the mucoid types. Direct agglutination tests showed no difference between the two types. The limit of agglutination was the same for each culture with the three serums. No difference was noted in the character of flocculation nor in the velocity of the reactions.

Reciprocal absorption tests were employed with the three cultures and their respective serums. The technic of Krumwiede (4) was followed except that absorption was carried out in 0.4 per cent salt

TABLE I.

*Agglutinin Absorption Tests with Normal and Mucoid Type Antiserums.**

Serum	Culture	Direct agglutination	Absorbing culture	Agglutination after absorption	Culture tested after absorption
Normal 922	Normal 922	1:51,200	Normal 922	1:200 1:200 1:200	Normal 922 Mucoid 1004 " 950
	Mucoid 1004	1:51,200	Mucoid 1004	1:400 1:400 1:400	Normal 922 Mucoid 1004 " 950
	" 950	1:51,200	" 950	1:400 1:400 1:400	Normal 922 Mucoid 1004 " 950
Mucoid 1004	Mucoid 1004	1:51,200	Mucoid 1004	1:800 1:800 1:800	Mucoid 1004 " 950 Normal 922
	" 950	1:51,200	" 950	1:800 1:800 1:400	Mucoid 1004 " 950 Normal 922
	Normal 922	1:51,200	Normal 922	1:400 1:400 1:400	Mucoid 1004 " 950 Normal 922
Mucoid 950	Mucoid 950	1:51,200	Mucoid 950	1:400 1:400 1:400	Mucoid 950 " 1004 Normal 922
	" 1004	1:51,200	" 1004	1:400 1:400 1:400	Mucoid 950 " 1004 Normal 922
	Normal 922	1:51,200	Normal 922	1:400 1:200 1:200	Mucoid 950 " 1004 Normal 922

* The absorbing dose was 1:5 and the serum dilution 1:10 throughout.

solution and final readings made after 3 hours of incubation at 37°C. The results of the absorption tests are given in Table I. No outstanding difference in absorptive capacity was displayed by the normal

and mucoid types of *B. paratyphi*. The variants were, however, a little less efficient in the removal of agglutinins from the three serums. The variation in absorptive capacity did not appear sufficiently great to indicate an actual antigenic difference between the two types.

TABLE II.

Preliminary Virulence Test with the Normal and Mucoid Types of B. paratyphi.

Culture	Dilution	Result	Spleen culture
Normal 922	1:2,000	Died 4 days	+
	1:20,000	" 10 "	+
	1:200,000	" 16 "	+
Mucoid 1004	1:2,000	" 10 "	+
	1:20,000	Killed 21 "	—
	1:200,000	" 21 "	+
" 950	1:2,000	Died 10 "	+
	1:20,000	Killed 21 "	—
	1:200,000	" 21 "	+

TABLE III.

Final Virulence Test with the Normal and Mucoid Types of B. paratyphi.

Culture	Dilution	Result	Spleen culture
Normal 922	1:200,000	Died 5 days	+
	1:200,000	" 6 "	+
	1:200,000	" 14 "	+
	1:200,000	Killed 26 "	+
Mucoid 1004	1:200,000	" 26 "	+
	1:200,000	" 26 "	+
	1:200,000	" 26 "	+
	1:200,000	" 26 "	—
" 950	1:200,000	Died 15 "	+
	1:200,000	Killed 26 "	+
	1:200,000	" 26 "	—
	1:200,000	" 26 "	+

A comparative study of the virulence of the normal and mucoid types was made on guinea pigs. As a preliminary graded dilutions

prepared from 18 hour bouillon cultures were injected intraperitoneally into guinea pigs of approximately 350 gm. weight. Little difference was noted in the number of bacteria per cc. with the three cultures. Plate counts gave roughly 600,000,000 bacteria per cc. for each. Dilutions of 1:2,000, 1:20,000, and 1:200,000 in 1 cc. of diluent were injected. One guinea pig only was injected with each dilution. The results of the test are given in Table II.

The preliminary test indicated a considerable difference in virulence between the two types. As a final check four guinea pigs were injected intraperitoneally with 1 cc. amounts of each culture in dilutions of 1:200,000, the highest lethal dose of the normal type. The results of the test are given in Table III.

The final test did not bear out the sharp difference in virulence indicated by the preliminary series. The normal type failed to kill 100 per cent of the test animals in the dilution employed. In addition one of the mucoid types displayed lethal action in that dilution. It seems probable that a difference in virulence does exist in favor of the normal type as the higher, but definite conclusions would be warranted only with a much larger series of animals.

The susceptibility of the mucoid variants to lysis by a filtrate active for the smooth type of *B. paratyphi* was determined. The filtrate employed was obtained from the feces of a mouse infected with paratyphoid through contact with a second mouse which had received *per os* administration of *B. paratyphi*. The method of serial dilution in bouillon was used in determining the activity of the filtrate. Dilutions ranging from 10^{-1} to 10^{-12} were made in 5 cc. amounts of bouillon and 0.05 cc. of an 18 hour bouillon culture added to each tube of the series. Readings were made at the end of 3, 6, and 48 hours incubation at 37°C. The end-point was determined by filtering the three tubes of highest dilution and retesting in series. The filtrate was active for its homologous culture, *i.e.*, the smooth type of *B. paratyphi*, through a dilution of 10^{-10} or 10^{-11}. Complete lysis of the culture never occurred. After 3 hours incubation there was no visible turbidity in dilutions of 10^{-1}, 10^{-2}, and 10^{-3}; from 10^{-4} through 10^{-6} there was a graded turbidity, and from 10^{-7} through 10^{-11} the turbidity was in the same as that of the control. After 6 hours incubation there was a faint turbidity in the first three dilutions which increased

in intensity upon further incubation. Resistant and susceptible bacteria could be isolated from any of the tubes in which the filtrate was active. The filtrate was inactive for the mucoid types. There was no inhibition of growth in any dilution. After 3 hours incubation all the tubes showed a distinct, finely floccular growth identical with that of the control. Further incubation resulted only in increased growth normal in appearance for the mucoid type. Plates poured and streaked at varying intervals of growth showed only normal mucoid colonies.

<div align="center">DISCUSSION.</div>

The guinea pig strain of B. paratyphi associated with the epidemic and regarded as the normal type appears to differ from the normal strains of human origin in that it displays a less marked tendency towards variation. Other workers have shown that the human strains commonly give rise to variants of the mucoid and rough types. Variation may occur under normal conditions of growth or it may be stimulated by prolonged cultivation and by rapid serial transfer. Alteration in the chemical nature of the medium by the addition of specific antiserum and by increased sodium chloride, peptone, or H ion concentration may also afford a stimulus to variation. The action of a specific lytic principle may likewise result in variation. The normal guinea pig strain has shown no tendency towards a mucoid variation under normal conditions of growth nor under the stimulus of specific antiserum, increased peptone concentration, or the action of a lytic principle. The latter stimuli do, however, initiate a variation resulting in the appearance of the rough type of variant which flocculates spontaneously in bouillon and is resistant to lysis.

Mucoid variants have not been recovered experimentally from the normal guinea pig strain either under parasitic or saprophytic conditions of growth. They have been recovered, however, from the guinea pig in two cases of spontaneous infection. The mucoid types have been compared with the normal strain as to biological characters. In general they display some difference in character from the mucoid types isolated by other workers from the human strains of B. paratyphi. The variants of the latter strains were usually found to be less agglutinable than the normal type and to show a tendency

towards reversion upon cultivation. The variants under discussion display little deviation from the normal type in agglutinability or in antigenic properties. The mucoid character of growth appears constant and no tendency towards reversion is displayed.

Experimental study has failed to demonstrate a stimulus which might account for the appearance of the mucoid types in the guinea pig host. If variation within the guinea pig organism resulting in a splitting from the normal type under the stimulus of resistance were accountable, it would seem that the mucoid types should be more frequently encountered. It is believed, rather, that the two types had a more remote, common ancestry and that the mucoid type as a variant of permanent characters was transmitted to the guinea pig population together with the normal type. Such an explanation likewise fails to account for the infrequency of its occurrence. Differences in virulence might be regarded as an associated factor.

SUMMARY.

The biological characters of two mucoid variants of *B. paratyphi* isolated from guinea pigs have been studied and compared with those of the normal type. The possible origin of the mucoid type is discussed.

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VIBRIOS FROM CALVES AND THEIR SEROLOGICAL RELATION TO VIBRIO FETUS.

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Nothing is known of the life cycle of *Vibrio fetus* beyond its presence in the bovine placenta and the fetus following abortion. In the digestive tract of the fetus the vibrio is usually found in pure culture. If the fetus is not invaded, however, the difficulties encountered in the placenta and uterine discharges, owing to contamination with miscellaneous saprophytic bacteria, have made isolation thus far impossible. This is due to its early very feeble multiplication, restricted to sealed agar tubes containing blood or fresh tissues or to tubes in an atmosphere containing a small per cent of CO_2, and its rapid destruction following injection into laboratory animals. The occasional encounter of vibrios in the intestinal tract of young calves calls attention to a possible locus of *Vibrio fetus*. These vibrios might be survivors of a fetal infection with *Vibrio fetus*, or they might represent a different group possibly associated with intestinal inflammation in calves after the 1st week.

The first strain (No. 174) was obtained in pure culture from the spleen of a calf killed when 10 days old. This animal began to scour when 5 days old and when killed was very weak. At autopsy, the middle portion of the small intestine was deeply congested. Fresh villi under the microscope showed the entire capillary network injected and certain groups of epithelial cells undergoing fatty changes. The large intestine contained normal fecal matter but the mucosa was overlaid with stringy elastic masses of mucus. *Bacillus fluorescens* was isolated from the intestines on agar plates. No special search was made for vibrios. In sections of small and large intestines, vibrios were not found. Agglutination relations of this strain have been briefly described.[1] Sera prepared with three different living cultures of *Vibrio fetus* failed to act upon this strain, although

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[1] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

clumping *Vibrio fetus* almost completely at 1:640 dilution. A specific serum prepared with the calf strain clumped it at 1:2,560 completely but failed to clump fifteen strains of *Vibrio fetus* at 1:20.

A second vibrio (No. 321) was isolated from a calf dead when 58 days old and noticed sick for about a week. The autopsy showed small ulcerations of rumen and the traces of early hemorrhages of the fourth stomach mucosa.[2] There was considerable mucus in the small intestine and the large intestine was congested. There was pneumonia of the left lung indicated by numerous, small, partly coalescing foci of a flesh red color. In sections these foci consisted of injected capillaries and alveoli filled with polymorphs. There was a large amount of coagulable protein in the urine.

The vibrio appeared in cultures of the liver, and in a spleen culture of a guinea pig killed 7 days after inoculation with contents of duodenum. The serological relation of this strain to *Vibrio fetus* has been briefly given.[1] It agrees so far as direct agglutination goes with *Vibrio fetus*. Morphological and cultural distinctions could not be demonstrated.

The two strains described were isolated in 1917 and 1918. In 1925 an obscure disease of calves was brought to our attention. No study of the disease was made on the spot and the one suggestive fact reported was that the calves had been bedded in buckwheat hulls. Two calves, one living, one dead, were brought for examination.

No. 1206.—Ayrshire female, about 3 weeks old and weighing 64½ pounds. Dried feces on buttocks and tail. Kept under observation 15 days when the calf died. During this period it was alternately constipated and passing watery feces. The temperature fluctuated around 39°C. There were signs of pneumonia. Milk was taken in small quantities.

Autopsy.—The rumen distended during life was about twice normal size and filled with cut straw, buckwheat shells, and hair. There was some congestion of large intestine. The cephalic lobe of the right lung was consolidated, larger than normal, and permeated with numerous grayish foci, 2 to 10 mm. in diameter. The pneumonia was associated with a bipolar organism (*B. bovisepticus*). Cultures with bits of liver, spleen, and kidneys remained sterile with the exception of one liver tube which contained a vibrio

No. 1208.—Guernsey female, brought to the Department about 12 hours after death. Age, 23 days. Weight after death, 59 pounds. The report was that the calf had developed scours There was pneumonia of the cephalic and ventral lobes of the right lung similar in character to that of No. 1206, and the same bipolar type of organism was isolated. The intestines were more or less congested. Cultures of spleen, liver, and kidneys were negative like those of No. 1206 with

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[2] Florence, L., *Am. J. Dis. Child.*, 1922, xxiii, 132.

the same exception that in one containing liver tissue a vibrio appeared in culture obviously like that from No. 1206. Films and sections of the intestines did not show vibrios.

At this time a small number of strains of *Vibrio fetus*, some isolated recently, others several years before, were on hand. Strains 174 and 321 had died out. Cultural differences between Strains 1206 and 1208 and the fetal strains were limited to a freer, more rapid multiplication on agar slants of the former. Agglutination tests were therefore undertaken. The vibrio cultures studied included five fetal strains and two calf strains. The cultures were grown on agar slants sealed with sealing wax. The growth was used after 3 to 4 days incubation at 37°C. The immune sera were prepared by in-

TABLE I.

Cross-Agglutination between Living Fetal and Calf Strains in Serum of Rabbits Immunized with Living Cultures and Cultures Heated at 100°C. for 2 Hours.

Rabbit serum prepared with	Agglutination titer						
	Fetal strains					Calf strains	
	741	996	997	1149	1217	1206	1208
997 (living)	1:2,560	1:5,120	1:10,240	1:5,120	1:5,120	1:1,280	1:2,560
997 (heated)	1:20	1:5,120	1:2,560	1:2,560	1:2,560	1:20	1:20
1208 (living)	1:2,560	1:2,560	1:1,280	1:2,560	1:1,280	1:2,560	1:10,240
1208 (heated)	1:20	1:20	1:20	1:40	1:20	1:2,560	1:2,560

jecting rabbits intraperitoneally. The injections were made with living cultures and with cultures heated 2 hours at 100°C. Living cultures, standardized by the Gates instrument to disappear at 2.4 on the scale, were used for the agglutinating and absorbing suspensions. The absorbing dose used was 6 times the concentration as represented by 2.4 on the Gates scale, *i.e.* 6 times the agglutinating dose. Each serum was absorbed by the same dose of each culture so that the results are comparable.

The experiments include cross-agglutinations with sera prepared by injecting living and heated cultures and reciprocal absorption tests carried out between calf and fetal strains, between fetal strains, and between calf strains. In Table I, the various relationships be-

tween fetal and calf strains tested with the sera of rabbits treated with living and heated cultures are briefly summarized.

With one exception, the fetal strains present no differences among themselves when tested with "living"[3] or "heated" sera of fetal strains. This exception is No. 741. In the "heated" sera of 997 only a little agglutinin for 741 was present. When the fetal strains were acted upon by "living" and "heated" sera of calf strain 1208, only the "living" serum was found to contain agglutinins for them. The calf strain agglutinins were only slightly lower in the "heated" calf strain serum than in the "living" serum. When the calf strains were treated with "living" and "heated" sera of the fetal strain 997, it was found that the agglutinins common between calf and fetal strains were present only in traces in the "heated" serum. There was therefore no cross-agglutination between calf and fetal strains when tested in "heated" sera. This test defines two serological groups based on the habitat of the strains. It indicates the existence of a distinct heat-stable calf-group antigenic factor and a distinct heat-stable fetal-group antigenic factor.

Absorption tests were next tried with results as given in Table II. In making and interpreting the absorption results the statements of Krumwiede, Cooper, and Provost[4] on agglutinin absorption were taken into consideration. These are in substance that direct agglutination may not be a reliable index of the serological relationship of a bacterium. Absorption of common agglutinins cannot be a criterion for likeness but such an absorption may suggest groupings. To determine agglutinative identity it is necessary to demonstrate specific agglutinins. The reciprocal absorption test is therefore the ultimate method available for determining agglutinogenic likeness or unlikeness. A complete reciprocal reaction indicates identity and the absence of such reciprocal reaction indicates dissimilarity. Between these extremes there may be found all degrees of partial reciprocal reactions.

Table II illustrates the different types of reciprocal absorption reactions which occurred between calf and fetal strains, between

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[3] These abbreviated terms signify "sera from rabbits injected with living vibrios or vibrios exposed to 100°C. for 2 hours."

[4] Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 55.

individual fetal strains, and between individual calf strains. The readings given are those made after three successive absorptions. The results throughout each series were very similar, so that the examples tabulated cover all the different degrees of reciprocal absorption observed.

A certain serological difference between calf and fetal strains was

TABLE II.

Examples of Reciprocal Absorption Tests.

Serum	Absorbed by	Agglutinated by		Titer before absorption	
1. Between calf and fetal strains					
		1208	997	1208	997
1208 (calf)	997	1:5,120	1:20	1:10,240	1:1,280
997 (fetal)	1208	1:160	1:10,240	1:2,560	1:10,240
2. Between individual fetal strains (1149 and 997)					
		1149	997	1149	997
1149	997	1:40	1:20	1:1,280	1:640
997	1149	1:320	1:320	1:5,120	1:10,240
				(996 and 997)	
		996	997	996	997
996	997	1:640	1:40	1:5,120	1:5,120
997	996	1:320	1:320	1:5,120	1:10,240
3. Between individual calf strains					
		1208	1206	1208	1206
1208	1206	1:640	1:40	1:10,240	1:2,560
1206	1208	1:80	1:640	1:10,240	1:5,120

demonstrated by reciprocal absorption tests using sera prepared with living organisms. The results showed an absence of reciprocal absorptions and suggest distinct calf and fetal group antigenic factors. Thus by two methods serological differences according to *source* have been demonstrated.

Reciprocal tests between fetal strains acted upon by sera prepared with living cultures show partial absorptions and almost complete

reactions, indicating an essential identity of fetal strains but possibly some individual strain factor as well. Three strains are practically identical according to the reciprocal tests; namely 997, 1217, and 1149. Strain 996, on the other hand, shows more strain individuality. The 996 agglutinins after three successive absorptions by the other strains are not removed beyond a certain point, while the agglutinins for these other strains continue to be reduced. This indicates some individual strain antigenic factor in 996 which produced these specific agglutinins. The other fetal culture, 741, is an exceptional strain. In absorption experiments it fits into the fetal group, but it differs from the other fetal strains as already mentioned by failing to agglutinate in the 997 serum prepared with the heated culture (Table I).

TABLE III.

Absorption of Agglutinins between the Calf Strains in Sera Prepared with Living and Heated Antigens.

Serum prepared with 1208	Absorbed by 1206	Agglutination titer after absorption		Agglutination titer before absorption	
		1206	1208	1206	1208
Living culture	3 successive times	1:40	1:640	1:2,560	1:10,240
Culture heated at 100°C., 2 hrs.	3 " "	1:20	1:40	1:2,560	1:2,560

The reciprocal tests between the calf strains with sera prepared from living cultures show a definite relation between these strains and also a strain individuality. Each strain absorbs agglutinins for the other up to a certain point, but after three successive absorptions specific agglutinins still remain for the antiserum-producing strain while agglutinins for the absorbing strain are almost wholly removed.

Comparative absorption tests between the two calf strains have been made also with sera prepared with living cultures and cultures heated at 100°C. The results are summarized in Table III. These indicate that the individual strain factor is heat-labile. With the living culture antiserum 1208 repeated absorptions by 1206 left a residue of agglutinins for 1208, while agglutinins for 1206 were wholly removed; but with heated culture antiserum 1208 repeated absorptions by 1206 removed agglutinins for 1208 and 1206 to about the same degree. Thus in the heated culture antiserum there is no spe-

cific strain agglutinin corresponding to that in the living culture antiserum; the individual strain factor is therefore heat-labile.

The relation of the antigenic factors to flagellar and somatic agglutinins has not been demonstrated since no non-motile vibrio form has been isolated. However, since the vibrio strains all show some motility, flagellar and somatic antigens are assumed to be present in all cultures and they may or may not be the basis of the serological difference between calf and fetal strains. Previous work has shown that flagellar antigen in the group of paratyphoid bacilli is heat-labile and somatic antigen heat-stable. The common and the individual strain heat-labile, antigenic factors in the vibrios probably represent the flagellar portion of the antigen and the specific calf and fetal group factors the somatic portion.

In brief these results indicate that at least four types of antigenic factors exist among the vibrio strains studied and any one strain may contain three of these different factors. They may be designated as (1) common vibrio factor, (2) calf group factor, (3) fetal group factor, (4) individual strain factor. The cross-agglutination tests with sera prepared with living cultures give an indication of the common vibrio factor. The cross-agglutination tests with sera prepared from heated cultures and reciprocal absorption tests between calf and fetal strains demonstrate the distinct calf and fetal group factors. The reciprocal absorption tests between fetal strains and between calf strains suggest some individual strain factors. Comparison of results with sera prepared with living and heated cultures indicate that the common vibrio factors and the individual strain factors are heat-labile while the specific calf group and specific fetal group factors are heat-stable. This partial analysis of the antigenic factors demonstrates that the vibrios are of a complex antigenic nature. The serological tests further indicate a close relationship between but not an identity of the calf and the fetal strains.

SUMMARY.

The calf vibrios thus far studied include one strain serologically distinct from the fetal strains. The others are closely related to the fetal strains though not identical with them. The pathogenic characters of the calf vibrios, either as possible descendents of *Vibrio fetus*, or as independent factors in the production of enteritis have not been demonstrated.

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EXPERIMENTAL BONE MARROW REACTIONS.

II. Normoblastosis Produced by India Ink. The Influence of Pregnancy on This Phenomenon.

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In a previous investigation (1) it was found that a comparatively aplastic, fatal anemia was produced in rabbits by repeated injections of collargol. This was interpreted as the result of injury to those endothelial cells which are considered as the precursor cells of the erythrocytes and clasmatocytes.

The marked and rapid effect obtained with colloidal silver suggested that this substance exerted not only a mechanical but also a toxic action upon the widespread system of endothelial cells. It seemed desirable, therefore, to analyze the reaction of the blood and blood-forming organs to a comparatively inert substance in colloidal suspension. Carbon particles served the purpose, especially as it has been noted by various observers (2, 3) that carbon exhibits a special affinity for the endothelial cells. ,

Material and Methods.

As experimental animals twenty-one rabbits were used.

The investigation was begun with an India ink manufactured by F. Weber Co., Philadelphia. This product was found to exert a marked stimulation on the blood-forming organs. Therefore, in the attempt to isolate the factor in the ink possessing this stimulating effect on the bone marrow, experiments were made later with the filtrate of this ink, obtained by passing it through a Berkefeld filter W, thus removing practically all the carbon particles, as well as dialysate of the ink, obtained by suspending 100 cc. of the ink in a parchment bag in 50 cc. of distilled water, and allowing it to stand for 4 days. Other inks,—Higgins' Waterproof and General Drawing Inks, as well as Chin Chin and Pelican Waterproof Drawing Inks, manufactured by Günther Wagner, Hanover, were tried, in order to determine whether or not the effect produced was a property common to col-

loidal suspensions of carbon particles, obtained in the market in the form of drawing inks.

As the present investigation was intended as a control of a previous study with colloidal silver (1) the same experimental procedure has been employed, unless otherwise stated, *i.e.*, the substance used was diluted with equal quantities of distilled water, and injections were given intravenously every 2nd day as a rule, the dose varying from 2 to 10 cc. The changes in the blood were followed by examinations at frequent intervals. Zenker-fixed tissues, stained with eosin and methylene blue, were used for microscopical examinations. Counts were also made on supravital bone marrow preparations, as well as on fixed smears of the bone marrow.

The nomenclature of the blood cells used in this paper is the one employed by Sabin (4). In fixed smears the difficulty encountered in classifying the earliest blood cells designated by many as hemoblasts, is fully appreciated, but it is felt it can be done with reasonable certainty. In the tables, the very early erythroblasts, with characteristic nuclei but no demonstrable hemoglobin have been classified separately, to show to what extent these forms appeared in the peripheral blood. No cells that could be classified as definite megaloblasts were found in the peripheral circulation although present in all the hyperplastic marrows.

EXPERIMENTAL OBSERVATIONS.

Injections of Weber's Drawing Ink.

Most of the observations here reported are based on the intravenous injections of F. Weber Co.'s Waterproof Drawing Ink and its filtrate. The results obtained with this ink were remarkably constant, if one allows for small variations due to individual differences in the animals studied.

With large doses (6 to 10 cc. of a 50 per cent suspension) the peripheral blood showed the following changes: first a gradual decline often preceded by a slight rise of erythrocytes and hemoglobin. If the same dose was used at each injection, this decline was usually followed by a rise to practically the initial level. With gradually increasing doses it was possible to produce a moderate anemia in three instances, and in one (No. H-25), a severe anemia which ended fatally, with terminal counts of 1,420,000 erythrocytes, 18 per cent hemoglobin, and 18,000 nucleated red cells per c.mm. This animal was given 54 doses, gradually increased from 4 to 10 cc., and administered with increasing frequency, over a period of 114 days.

When smaller dosage was employed such as 4 cc. of a 50 per cent

solution, the hemoglobin and erythrocytes increased above the initial level. This increase was sustained for a considerable but variable time, then, with continued injections, the red blood cells and hemoglobin fell to, or below, the initial level. In one animal (No. H-60) receiving 4 cc. doses, the erythrocytes and hemoglobin gradually

TABLE I.

Rabbit H-24. Intravenous Injections of Weber's Drawing Ink Diluted with an Equal Quantity of Distilled Water. Large Dosage.

Date	Hemo-globin	Red blood cells	White blood cells	Normo-blasts	Erythroblasts		Remarks
					With hemo-globin	No hemo-globin	
			per c.mm.				
1924	*per cent*						
Dec. 10	57	5,780,000	12,000	0	0	0	Injections begun
" 15	53	5,760,000	24,000	6,330	0	0	Dose 6 cc. three times a wk.,
" 17	48	4,730,000	9,400	16,650	273	0	except where otherwise
" 22	52	4,250,000	11,000	4,990	0	0	noted
" 27	47	3,610,000	13,400	20,880	286	0	
1925							
Jan. 1	46	4,230,000	18,000	12,200	200	0	
" 6	50	4,490,000	7,200	13,830	87	0	Dose 8 cc. injections given
" 10	55	5,310,000	15,900	38,790	954	0	every day from Jan. 5 to
" 15	49	3,890,000	14,000	54,660	8,410	0	15
" 20	48	4,870,000	5,500	37,760	3,360	60	
" 27	48	4,600,000	12,300	31,720	3,250	0	
Feb. 2	45	4,260,000	16,600	39,360	3,600	0	
" 10	44	4,270,000	13,100	11,310	130	0	
" 17	52	4,380,000	16,000	19,140	1,320	0	
" 25	52	4,390,000	23,000	35,220	3,690	246	
Mar. 2	54	4,620,000	12,500	3,430	0	0	Dose omitted Feb. 28
" 5	57	5,180,000	15,700	22,360	1,204	0	Killed

declined to about 50 per cent of normal, then a gradual increase was apparent especially in the hemoglobin.

The most striking and constant feature in all the animals studied, with large or small dosage, was the appearance, after one or two injections, of nucleated red blood cells in the peripheral blood. The number of nucleated cells increased as injections were continued, and in several instances reached high values (60,000 to 70,000 per c.mm.).

In the beginning of the course the nucleated red cells were primarily normoblasts; later however, erythroblasts of increasingly early stages appeared in the peripheral blood. This outpouring of nucleated red blood cells continued uninterrupted during the entire time that the animals were under observation, in two instances for 4 months. The

TABLE II.

Rabbit H-58. Intravenous Injections of Weber's Drawing Ink Diluted with an Equal Quantity of Distilled Water. Small Dosage. Illustrating Effect of Pregnancy.

Date	Hemo-globin	Red blood cells	White blood cells	Normo-blasts	Erythroblasts		Remarks
					With hemo-globin	No hemo-globin	
			per c mm.				
1925	*per cent*						
Sept. 29	78	6,770,000	13,900	0	0	0	Injection begun
Oct. 2	83	6,970,000	9,130	4,473	91	0	Dose 4 cc. three times a wk.
" 6	104	7,470,000	9,000	630	0	0	
" 10	92	6,980,000	5,900	12,962	236	0	
" 15	101	7,100,000	11,080	14,520	1,100	0	
" 20	80	6,890,000	5,600	12,840	560	0	
" 24	93	6,730,000	12,500	27,375	750	0	
" 29	87	7,370,000	17,600	24,288	1,232	0	
Nov. 3	92	7,040,000	10,240	8,058	102	0	One injection omitted Nov. 6
" 9	83	6,220,000	13,000	390	0	0	
" 17	71	5,130,000	8,300	0	0	0	
" 19	74	5,240,000	10,990	110	0	0	Litter of four young
" 24	70	5,280,000	12,440	10,788	3,968	248	Nov. 19. Blood count
Dec. 3	64	5,370,000	11,200	27,360	5,472	342	done after litter cast
" 11	63	5,900,000	18,200	18,982	1,162	581	
" 22	70	6,380,000	15,100	8,200	0	0	
" 31	80	5,640,000	17,000	11,400	1,900	0	
1926							
Jan. 12	78	5,900,000	14,000	7,348	668	0	
Feb. 2	73	5,540,000	33,500	8,650	2,422	346	
" 16	89	8,040,000	23,200	7,860	975	245	Killed

magnitude of this reaction varied directly with the size of the dose and the frequency of the administration. The general course of one of the animals given large doses is illustrated in Table I.

In a female which became pregnant, the appearance of nucleated red blood cells in the peripheral blood was markedly depressed for

about 2 weeks, and finally completely suppressed, before the litter was cast, only to be resumed with renewed vigor after the termination of the pregnancy. This observation was verified in another animal allowed to become pregnant during the course of the experiment. In Table II is illustrated the effect of comparatively small dosage, and the influence of pregnancy on the blood picture. It is of interest to note that the appearance of nucleated red blood cells was often concomitant with an increase of the erythrocytes and hemoglobin in healthy animals above the supposedly normal initial level. That the number of nucleated red cells in the peripheral blood represented an actual stimulation of the erythrocytic centers and not a release only of already formed cells in the marrow is evidenced by (1) the length of time that the animals exhibited this phenomenon (one animal 141 days), (2) the microscopic findings of the bone marrow.

The bone marrow in all the animals receiving injections of Weber's Drawing Ink showed a marked hyperplasia. The degree of hyperplasia varied with the length of time of administration and the size of the dose. In those animals receiving large doses for a comparatively short time the tendency seemed to be toward less replacement of fat, while with smaller dosage, the decrease of fat cells was more marked. The hyperplasia was largely limited to cells of the erythrocyte series and normoblasts predominated. When a few small doses were given, younger forms were more evident in the hyperplastic areas. In animals injected over a long period of time, the bone marrow was always hyperplastic in structure, and this hyperplasia included also the leucocyte series. In these cases there was also evidence of a patchy hypoplasia. This was especially marked in No. H-60, in which the patchy hypoplasia was adjacent to areas of most intense erythrocyte hyperplasia. In addition many clasmatocytes, scattered throughout in all the sections, were filled with great quantities of carbon particles, in many instances obscuring the finer architecture of the bone marrow. A striking feature in the bone marrows studied was the absence of the endothelial wall in many of the venous sinusoids. Under low magnification the mature red cells were confined to definite channels, the boundary line of which, under higher magnification, proved to be nucleated red cells in various stages of maturation. Occasionally a large swollen endothelial cell would be in line with a megaloblast or

erythroblast, furnishing part of the boundary line of the blood vessels, but more frequently the boundary consisted of erythroblasts and normoblasts, many of them apparently loose and mixed with mature red cells. This indicates that in these hyperplastic marrows, red blood cell formation was taking place intravascularly, and, as far as could be determined, from the vascular endothelium of patent, functioning sinuses. The same observations have been made by Doan, Cunningham, and Sabin (5).

Other points of interest noted in the microscopic sections were the large number of nucleated red cells in the spleen; the practical absence of carbon particles from the retroperitoneal mesenteric lymph nodes; and the considerable accumulation of carbon particles in the lung. Whether the nucleated red blood cells were taken out of the blood stream or formed locally in the spleen could not be determined.

Injections with Dialysed Weber's Drawing Ink and the Dialysate.

In order to isolate, if possible, the factor producing this stimulation of the erythrocytic centers described above, the ink was dialysed through a parchment membrane in running water. If the process of dialysation was continued more than 16 hours, a gelatinous mass was formed which was unfit for intravenous injections. The results obtained with ink dialysed between 12 and 16 hours gave essentially the same, but less striking results than those obtained with the untreated ink.

The dialysate of the ink, obtained by suspending 100 cc. of the ink in a parchment bag in 50 cc. of distilled water and allowing it to stand for 4 days, when injected intravenously seemed to be without any effect on the blood. The bone marrow of the animal injected was essentially normal.

Injections with Filtrate of Ink.

The filtrate obtained by passing the ink through a Berkefeld filter W gave results similar to the whole ink injected intravenously, and, as far as could be determined, by removing the ink particles the action on the hematopoietic organs was somewhat enhanced. In Table III, the course of one animal so injected is illustrated. The dosage in this animal was 4 cc. of the undiluted filtrate, comparable to an 8 cc. dose

given to Rabbit H-24.   In all the animals the initial rise was followed
with a marked drop in the hemoglobin and erythrocytes.   Both the
rise and the anemia following were accompanied by tremendous out-
pourings of nucleated red cells, the number of which declined some-
what after reaching a maximum.   This decline was usually accom-
panied by a rise in hemoglobin and erythrocyte values.   Efforts

TABLE III.

*Rabbit H-63.   Intravenous Injections of Filtrate of Weber's Drawing Ink.   Ink
Passed through Berkefeld Filter W.   Large Dosage.*

Date	Hemo-globin	Red blood cells	White blood cells	Normo-blasts	Erythroblasts With hemo-globin	Erythroblasts No hemo-globin	Remarks
					per c.mm.		
1925	*per cent*						
Oct. 15	80	6,280,000	14,800	0	0	0	
" 19	79	6,140,000	10,800	0	0	0	
" 20			24,500	0	0	0	Injections begun. Dose 4
" 23	99	6,700,000	23,110	7,590	0	0	cc. three times a wk. White
" 27	96	6,090,000	12,670	6,220	0	0	blood cell count 1 hr. after
" 31	75	5,590,000	9,950	10,240	199	0	first injection
Nov. 5	72	5,890,000	28,920	11,630	290	0	One injection omitted Nov. 6
" 9	57	5,110,000	9,080	5,720	0	0	
" 17	58	4,510,000	7,900	41,310	2,142	0	
" 24	58	4,770,000	15,050	21,190	2,558	0	
Dec. 3	45	3,990,000	16,510	63,630	4,209	0	
" 12	46	3,530,000	24,000	25,540	11,830	258	
" 22	45	3,400,000	20,100	39,760	8,644	864	
" 31	42	3,350,000	21,700	64,900	11,694	1,222	
1926							
Jan. 12	50	2,780,000	14,500	61,230	16,329	1,134	
Feb. 2	52	3,330,000	28,800	41,920	9,600	320	Last injection Feb. 1
" 3	48	3,200,000	21,900	28,930	8,775	325	Killed

were made to exhaust the marrow completely by increased dosage
and more frequent administration, but without success.   The struc-
ture of the marrow was similar to that obtained from animals injected
with the whole ink with this difference; that the clasmatocytes filled
with ink particles were replaced by enlarged clasmatocytes staining
pale red with eosin, usually appearing homogeneous with occasional
vacuoles and granular inclusions.

Injections with Higgins' India Ink.

Five animals were injected with Higgins' India Ink, three with the Waterproof Drawing Ink and two with the General Drawing Ink. The dose in all instances was 4 cc. of a 50 per cent suspension, and the number varied between 4 and 34 injections administered every 2nd day. The course of these animals was identical in the main with the results reported by Sapinoso, Berg, and Jobling (6), *i.e.*, first there was a decline of red blood cells and hemoglobin, followed by a rise to the initial level. Nucleated red cells in the normoblastic stage appeared in the peripheral blood, but the magnitude of the reaction was slight when compared with those obtained with Weber's India Ink and its filtrate. At no time were more than 2280 normoblasts found per c.mm. The most striking difference, however, was found in the bone marrow. The carbon was deposited in clasmatocytes to a great extent, but considerable amounts were found in the endothelium lining the dilated blood vessels. There was little or no hyperplasia of the bone marrow and the cells present were mainly of the leucocyte series.

The enormous capacity of the endothelial cells to form clasmatocytes and phagocyte particulate matter is realized when studying sections from animals injected over long periods of time, provided that these particles or colloidal suspensions are of an inert nature. The findings above prove conclusively that carbon particles as such stimulate the endothelial cells mainly toward clasmatocyte formation. With colloidal silver on the other hand, there seems to have been a toxic effect on the endothelial cells incapacitating the entire system very rapidly. This may perhaps explain the divergence of results by investigators "blocking" the so called "reticulo-endothelial system." The colloid or particulate matter used may be inert; it may stimulate or it may depress this widespread system of cells.

Injections with Günther Wagner's India Ink.

Two brands of German manufacture were used, namely, Chin Chin and Pelican Waterproof Ink. The results were essentially the same as with Weber's India Ink, except that the changes of both the peripheral blood and blood-forming organs were less marked.

DISCUSSION.

Summarizing the results obtained by the injection of various India inks, certain features ought to be emphasized. With Weber's India Ink a tremendous outpouring of nucleated red cells occurred which, in the light of the experiments described above, may be attributed to a substance which passes through a Berkefeld filter and seems to be non-dialysable. This substance, apparently a protective colloid, appears to have essentially the same effect on the blood and the blood-forming organs, whether it is associated with the ink or separated from it. With large doses after an initial rise of erythrocyte and hemoglobin values, there was a decrease in these constituents of the peripheral blood, while with smaller dosage there was mainly an increase of red cells and hemoglobin sustained for a variable time and followed later by a decrease. Associated both with the rise and fall in red cells there was an outpouring of nucleated red cells, as a rule proportional to the size of the dose and the number of administrations. The interesting feature, however, is the delivery of the nucleated red cells in the peripheral blood in the face of apparently an adequate number of circulating red cells.

The conditions in which nucleated red cells appear in the peripheral blood were reviewed by Drinker, Drinker, and Kreutzmann (7, *a*) in 1918. Except in anemia due to various causative factors, nucleated red cells do not appear in the peripheral blood save as a result of marrow disintegration (8), as an occasional concomitant of leucocytosis (9, 10), and, very rarely, in animal and man subjected to sudden low barometric pressure (11). The crowding out of the nucleated red cells in acute myelogenous leucemia and in metastatic tumor growth in the marrow (12) is easily explained as pressure phenomena.

With a normal marrow the appearance of nucleated red cells in the peripheral blood stream can in all probability be explained by growth pressure (7, *b*). This pressure may be local and does not necessitate an extensive hypertrophy throughout the tissues. If the picture of the bone marrow from the histological and physiological points of view is kept in mind, the appearance of nucleated red cells in the peripheral blood when the marrow is stimulated becomes apparent. Histologically, we have a tissue with sinuses and intersinusoidal capil-

laries situated between the fat cells, and outside the functional blood stream are the marrow cells, the red cells, however, developing in the intersinusoidal closed, but potential, capillaries as has been shown by Doan, Cunningham, and Sabin (5). Occasionally in a hyperplastic marrow the developing red blood cells may be situated directly in the path of the blood stream as seen in these experiments. Physiologically on the other hand the bone marrow is a highly sensitive tissue confined to an inexpansible environment with all of its elements incompressible, the path of least resistance being the blood stream, as has been pointed out by Drinker (7, c).

In the experiments described the appearance of the unprecedented number of normoblasts and erythroblasts in the peripheral blood can therefore be explained by growth pressure. The growth pressure may be produced in this instance by two factors, the most important one being the marked increase of the nucleated red cells in the bone marrow; the other being the number of large clasmatocytes scattered through the bone marrow and encroaching to a considerable extent on the limited space. With this pressure from developing erythrocytes and clasmatocytes the intersinusoidal capillaries may be forced open prematurely, allowing the circulating blood to wash over the immature red cells; or the red cells may develop primarily on the edges of the already open sinuses. Growth pressure due to a considerable increase of the clasmatocytes in the bone marrow may perhaps explain the moderate number of normoblasts found in the peripheral blood of animals injected with Higgins' India Ink, the bone marrow of which showed little or no hyperplasia of the erythrocyte series but a great many clasmatocytes.

As an explanation of the delivery of red cells in the peripheral blood stream, disintegration of the marrow must be thought of, but can be dismissed at once because of the length of time the animals exhibited this phenomenon, the content of the peripheral blood, and the histological picture of the bone marrow. It is of course conceivable that the stickiness of the red cells except the mature erythrocytes, as has been pointed out by Key (13), may be lessened or abolished in some way by the substance used for injections, but this does not explain adequately the phenomena encountered.

The marked suppression of the delivery of nucleated red blood cells

in the blood stream, concomitant with a slight fall in erythrocytes and hemoglobin in the course of pregnancy, I do not attempt to explain, but it is an interesting fact showing the dominant effect of pregnancy on the bone marrow over an agent which otherwise did not fail to elicit this characteristic reaction.

It is evident that from the experiments above no definite conclusions can be drawn as to the nature of this bone marrow stimulus and whether it operates directly on the bone marrow or indirectly through some other organs. Investigation of the subject, however, is now in progress.

CONCLUSIONS.

1. Intravenous injections in rabbits of F. Weber Co.'s Drawing Ink gave the following results:

(a) With large doses, an outpouring of nucleated red blood cells, in many instances reaching high values (60,000 to 70,000 per c.mm.), accompanied by a slight initial rise of erythrocytes and hemoglobin, followed by a moderate anemia and in one animal, a severe anemia.

(b) With smaller doses the presence of nucleated red cells in the peripheral blood was correspondingly less, the hemoglobin and erythrocytes increasing above the initial level. This increase was sustained for a considerable time, followed by a decrease to or below the initial level.

(c) Pregnancy caused a marked suppression, and, shortly before delivery, a complete suppression of the normoblastosis. Nucleated red blood cells reappeared in the blood after the litter was cast.

2. The filtrate of Weber's Drawing Ink, obtained through a Berkefeld filter W, produced the same effects, somewhat enhanced, as the whole ink.

3. The bone marrow of the animals injected with Weber's ink and the filtrate, both showed a marked erythrocytic hyperplasia, with many open sinuses lined with nucleated red blood cells in all stages of maturation.

4. As the dialysed ink gave practically the same results, and the dialysate proved to be without any effect, the conclusion was drawn that a non-dialysable protective colloid was responsible for the marked stimulation of the hematopoietic organs.

5. The delivery of nucleated erythrocytes was interpreted as due to growth pressure induced by rapidly growing red blood cells as well as intrasinusoidal formation of erythrocytes. Pressure due to rapidly increasing phagocytic cells must also be considered.

6. It is fair to conclude that carbon particles as such stimulate endothelial cells mainly toward clasmatocyte formation without incapacitating the endothelial cells, while colloidal silver apparently had a toxic and incapacitating effect on this system of cells.

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LOCAL IMMUNITY IN EXPERIMENTAL ERYSIPELAS.

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PLATES 8 TO 10.

Evidence pointing to the production of a true local tissue immunity following local streptococcus infection has been reported by Gay and Rhodes.[1]   These authors used in their experiments on rabbits *Streptococcus pyogenes* ("H") originally obtained from a patient having empyema following an attack of measles.   In the experiments to be reported, strains of hemolytic streptococci cultivated from the skin of patients with facial erysipelas have been employed.

In this report, it will be shown that not only the areas of skin actually involved in the reaction attending the immunization are refractory to subsequent injections but that the field in the general direction of the lymph flow is likewise relatively resistant.

Methods.

The strains of beta hemolytic streptococci employed, were isolated from the advancing edge of the erysipelas lesion in human cases by injecting 0.2 cc. of beef infusion broth endodermally and after a half-minute aspirating as much of the injected liquid as possible.   The aspirated fluid was usually admixed with blood and a small drop of blood welled up from the track of the needle.   These fluids were mixed in the syringe and blown out on rabbit blood agar plates.

The isolated strains were kept on blood agar slants, and from time to time passed through rabbits by endodermal inoculation.

For the endodermal injection, the streptococci were grown on rabbit blood agar slants for 18 hours at 37°C. and washed off in beef infusion broth of pH 7.4.   1/10 of an agar slant growth suspended in 0.25 cc. of broth was the dose usually employed.

The hair over the surface to be injected was removed by clipping closely with sharp curved scissors.   Shaving and chemical depilation used in the earlier experiments have been found unsuitable.

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[1] Gay, F. P., and Rhodes, B., *J. Infect. Dis.*, 1922, xxxi, 101.

The Experimental Lesion.

The experimental lesion has been described by Rivers[2] who was concerned especially with the diffuse lesion occurring in about two out of ten rabbits injected. In a series of 60 rabbits in which various experiments on local immunity were made, we observed particularly the direction of spread of the lesion from the site of inoculation.

The characteristic redness and edema appeared in all but two animals. In these there seemed to be little defense reaction of the tissues as redness and edema were absent. The skin surrounding the site of inoculation became white and the area of white necrosis spread several cm. eccentrically. Both animals became acutely ill, and after 2 days of high fever, the temperature became subnormal and death occurred on the 3rd day. Hemolytic streptococci were obtained in pure culture from the heart's blood at necropsy.

In five other animals, receiving the usual dose, there was a definite reaction from 1×6 cm. to 6×6 cm. without the formation of pus; blood invasion and death occurred in from 2 to 7 days.

Pus formation in considerable quantity occurred in nine instances and in only small amounts in nine others. A frequent occurrence was the appearance of small white areas of skin necrosis which on superficial examination suggested pus formation. Such areas measuring from 2 to 10 mm. occurred eighteen times and in five other instances these areas attained a diameter of 6 cm. In three instances in which the Corley strain had been inoculated, the necrotic areas were hemorrhagic. In other instances, not included in these series, Strain B had produced similar lesions.

In general, the lesions may be divided into two classes according to the direction of spread; (1) downward (*i.e.*, ventrally) resulting in a long, narrow band of redness and edema measuring after 48 hours from 1 to 2 cm. in width and from 3 to 17 cm. in length, and (2) circular area of redness and swelling from 1 to 6 cm. in diameter. In the latter, the geometric centers were always below or ventral to the site of inoculation. The relative occurrence of the two types of spread in one series was 28 to 4, in the total number reported here, thirtynine of Type 1 and nineteen of Type 2. In two instances, there was

[2] Rivers, T. M., *J. Exp. Med.*, 1925, xli, 179.

first a circular spread in which the downward trend met some resistance and after 24 hours there was extension downward resulting in a reniform lesion.

Diffuse localized erythema occurred 4 times among the 60 rabbits on the first injection. In two other rabbits, in which this type of reaction was not present after the first injection, it did occur on one subsequent injection.

Diffuse generalized erythema of the skin has been encountered only twice among 300 rabbits receiving endodermal injection. Both of these rabbits were white without markings.

In the series of 60 rabbits, five rabbits died and showed adhesive pericarditis after several months' immunization.

Lesions Produced by Repeated Injections.

Cobbett and Melsome[3] failed to obtain erysipelas on reinoculation of the same ear of the rabbit during a period of from 22 to 100 days following the first successful inoculation. A second inoculation in the protected ear caused a transitory and limited infection. Gay and Rhodes[1] report protection against endodermal reinoculation enduring from 3 weeks to 3 months in rabbit skin after recovery from experimental erysipelas. These authors used an empyema strain of beta hemolytic streptococcus.

In the course of the production of monovalent immune serum in rabbits by repeated endodermal inoculation, records were kept of the extent of the lesion resulting from the injection of a standard dose at weekly intervals.

Whereas the spread after the first endodermal injection more often descended ventrally in the direction of the lymph flow rather than anteroposteriorly, after the second subsequent injections the resulting lesions were usually circular and eccentric to the point of injection. The circular lesions resulting from the first injection were compared with the same type following the subsequent inoculations in seventeen rabbits. The average maximum diameter after the first injection was 2.2 cm., after the second, 2.0 cm., the third, 1.7 cm., and the fourth, 0.8 cm.

[3] Cobbett, L., and Melsome, W. S., *J. Path. and Bact.*, 1896, iii, 39; *Centr. allg. Path. u. path. Anat.*, 1898, ix, 827.

Increased Resistance of the Skin on One Side.

In the course of immunization with various strains by the endodermal injection of living streptococci, some of the rabbits were by chance injected several times at intervals on only one side. After several injections, the reactions to which were successively smaller, an injection was made on the other side of the rabbit, with the result that the lesion was practically as large as after the first immunizing dose. The protocols of six experiments illustrating the difference in the response of the non-injected and the injected sides in partially immunized rabbits are shown below.

Experiment 1.

Rabbit 1.—Oct. 30, 1925. Hair removed from left side by clipping with scissors followed by shaving. Into two sites in depilated area was injected, endodermally, 0.4 cc. of broth containing 4/10 of the 18 hour growth of Strain Z on a rabbit blood agar slant. The resulting lesions were red and edematous, the larger of which was 1 × 5 cm. in extent.

Nov. 19. Hair removed from both sides. At two points in each depilated area there was injected 0.25 cc. of broth containing 2/10 of the 18 hour growth of Strain Z on a rabbit blood agar slant. The resulting lesions were 2 cm. in diameter on the left side and 3.5 cm. on the right.

Rabbit 2.—Oct. 3, 1925. Hair removed from left side by clipping with scissors, followed by shaving. Into the depilated area there were injected endodermally, (1) 0.4 cc. of broth containing 4/10 of the 18 hour growth of Strain Z on a rabbit blood agar slant, and (2) 0.8 cc. containing 8/10 of a similar culture of Strain C. Reactions consisted in edema and reddening 3 × 1.5 cm. with Strain Z, and 6 × 4 cm. with Strain C.

Oct. 30. Left side injected at one point with 0.5 cc. of broth containing 1/2 of the 18 hour growth of Strain C on a rabbit blood agar slant. Resulting lesion consisted in edema and reddening 3 × 5 cm. in area.

Nov. 11. Left side injected with 1 cc. containing 7/10 of the 18 hour growth of Strain C on a rabbit blood agar slant. Resulting lesion, measuring 1.5 × 3 cm., was very red and slightly elevated and indurated.

Nov. 19. Hair removed from both sides. Each side received an injection of 0.4 cc. of broth containing 4/10 of the 18 hour growth of Strain C on a rabbit blood agar slant. Lesion: on left was 2.5 cm., on right, 5 cm. in diameter.

Rabbit 3.—Oct. 3, 1925. Hair removed from left side by clipping with scissors, followed by shaving. Into depilated area there was injected endodermally 0.4 cc. of broth containing 4/10 of the 18 hour growth on a rabbit blood agar slant of Strain P. The resulting lesion, measuring 3 cm. in diameter, was red and edematous.

Oct. 30.   0.5 cc. of broth containing 1/2 of the 18 hour growth of Strain P on a rabbit blood agar slant was injected endodermally on the same side.   The resulting lesion was 2 cm. in diameter.

Nov. 19.   There was injected endodermally on both sides 0.2 cc. of broth containing 2/10 of the 18 hour growth of Strain P on a rabbit blood agar slant. Resulting lesion, left side, slight reddening and edema 1 cm. in diameter; right side, marked edema and reddening 3 cm. in diameter.

Rabbit 4.—Sept. 28, 1925.   Hair removed from left side by clipping followed by shaving.   Into the depilated area, there was injected endodermally 0.5 cc. of broth containing 1/2 of the 18 hour growth of Strain P on a rabbit blood agar slant.   Result: induration and reddening 1 cm. in diameter.

Nov. 12.   0.25 cc. of broth containing 1/4 the 18 hour growth of Strain P on a rabbit blood agar slant was injected endodermally.   There was slight reddening and edema, after 24 hours.

Nov. 19.   0.2 cc. of broth containing 2/10 of the 18 hour growth of Strain P on a rabbit blood agar slant was injected endodermally on each side.

Resulting lesion on left side, slightly reddened papule 0.5 cm. in diameter; right side, intense reddening 2 cm. in diameter.

Rabbit 5.—Sept. 28, 1925.   Hair removed from left side by clipping with scissors followed by shaving.   Into the depilated area, there were injected endodermally (1) 0.5 cc. of broth containing 1/2 of the 18 hour growth of Strain B on a rabbit blood agar slant and (2) an equal dose of Strain Z.   Resulting lesions from Strain B, reddening and edema 3.5×1.5 cm.; from Strain Z, reddening and edema 1 cm. in diameter.

Nov. 12.   0.4 cc. of broth containing 4/10 of the 18 hour growth of Strain B on a rabbit blood agar slant was injected endodermally at two points on left side. Resulting lesions, reddening and edema 1×3 and 3×3 cm.

Nov. 19.   0.2 cc. of broth containing 2/10 of the 18 hour growth of Strain B on a rabbit blood agar slant was injected at one point on each side.   Resulting lesion, left side, reddening and edema 1.5 cm. in diameter; right side, 4.5 cm.

Experiment 2.

Rabbit 6.—Sept. 28, 1925.   Hair removed from left side by clipping followed by shaving.   Into the depilated area there was injected endodermally 0.5 cc. of broth containing 1/2 of the 24 hour growth of Strain H on a rabbit blood agar slant. Resulting lesion: reddening and induration 2 cm. in diameter.

Nov. 11.   0.4 cc. of broth containing 4/10 of the 18 hour growth of Strain H on a rabbit blood agar slant was injected endodermally on the left side.   Resulting lesion: induration and intense reddening with skin necrosis 2 cm. in diameter.

Nov. 21.   0.25 cc. of broth containing 1/4 of the 18 hour growth of Strain H on a rabbit blood agar slant was injected endodermally at two points on each side. Resulting lesions, left, reddening and edema 1 cm. in diameter; right, reddening and edema 1.5 and 3 cm. in diameter.

Extent of Local Immune Area.

The skin previously invaded by the erysipelas lesion is markedly resistant to subsequent infection with strains of the erysipelas group.

Since the spread of the lesion is usually downward, or ventral to the point of injection, it is possible that areas in the direction of the lymph flow may also become resistant even though such areas may not be visibly involved in the erysipelas lesion. To test this possibility, injections of the same dose were made on the same day at various points into the skin surrounding the healed area in partially immunized rabbits. The points of injection were usually (1) ventral to the edge of the previous area involved, (2) anterior or proximal, and (3) on the other side of the rabbit where no previous injections had been made.

The results of these injections show definitely that areas in the direction of the lymph flow though not within the previously involved area are refractory to the injections of the homologous strains. The protocols follow.

Experiment 3.

Rabbit 5.—Immunized against Strain B. Injections begun Sept. 28, 1925, and repeated once a week.

Dec. 7. Injected endodermally with 0.2 cc. containing 2/10 of the 18 hour growth of Strain B on a rabbit blood agar slant at each of the following points equidistant from the old lesion: (1) ventral to the site of the old lesion; (2) dorsal to it; (3) anterior to it.

Resulting lesions were respectively, 1.5 cm. in diameter, 3 × 2 cm., and 2. 5 × 0.5 cm.

Rabbit 2.—This rabbit had received four endodermal injections of 4/10 of the culture of Strain C, well separated on the left side at weekly intervals during the preceding 4 weeks. The last injection was on Dec. 7, 1925. On Dec. 14, three endodermal injections each of 4/10 of a culture of Strain C in 0.4 cc. broth were made at three different points:

(1) 1 cm. below the edge of the lesion of Dec. 7; (2) 1 cm. anterior to the same lesion; and (3) at a corresponding point on the right side.

The maximum measurements at the three sites were 1 cm., 2.5 cm., and 5 cm., respectively. Photographs of these lesions are shown in Figs. 1 and 2.

Experiment 4.

Rabbit 7.—Dec. 1, 1925. Hair removed from left side by close clipping. Into two sites in depilated area was injected endodermally 0.25 cc. of broth containing

2/10 of the 18 hour growth of Strain B on a rabbit blood agar slant. Resulting lesions measuring 2 × 4 and 2 × 5 cm. after 24 hours were red and edematous with small central patches of skin necrosis.

Jan. 29, 1926. The same dose of living organisms (Strain B) was injected endodermally at two points on the left side, one below site of old lesions and the other adjacent to it, and at two points on the right side. Resulting lesions, left side, below site of first inoculation, a small red swelling 0.5 × 2 cm. in area, with a patch of skin necrosis at center; adjacent lesion, red and edematous, 1.5 × 3 cm. Right side, reddening and edema and slight skin necrosis 1.5 and 3 cm. in diameter.

Rabbit 8.—Injected on same dates and with same doses as Rabbit 7, but the organism used was Strain C. First injections on left resulted in two areas of reddening and edema, 3 × 5 cm. in size. After the second series of injections, the lesion below the old site of inoculation consisted in a red papule 1 cm. in diameter. Adjacent to it, the lesion was 1 × 3 cm. in size and showed very little swelling. On the other side, the lesions consisted in reddening and edema 2 × 3 cm. in area.

Experiment 5.

Rabbits 9 ⎱ Apr. 15, 1926. Hair removed from left side by close clipping with
        10 ⎰ scissors. Into depilated area was injected endodermally 0.2 cc.
        11 ⎰ of broth containing 1/500 of the 18 hour growth on a rabbit blood agar slant of the following strains:

Rabbit 9 received Strain P; Rabbit 10, Strain B; Rabbit 11, Strain C.

Resulting lesions after 24 hours: Rabbit 9, reddening and intense edema 1.5 × 2 cm.

Rabbit 10: Edema and reddening 2 × 3.5 cm.

Rabbit 11: Edema, reddening, and large patch of skin necrosis 2 × 6.5 cm.

May 22. Hair removed from both sides by clipping. The three rabbits were injected with their respective strains at four points with 0.2 cc. of broth containing 1/500 of the 18 hour growth on a rabbit blood agar slant, as follows:

1. Left side: Below old lesion.
2. " " On site of old lesion.
3. " " To the side of old lesion.
4. Right side.

Resulting lesions after 24 hours:

Rabbit 9: left side, below old lesion, edema and reddening 1.5 cm. in diameter. On site of old lesion, slight edema and reddening 0.8 cm. in diameter. To the side of old lesion, edema and reddening 1.8 cm. in diameter. Right side, edema and reddening 2 × 2.3 cm. in diameter (Figs. 3 and 4).

Rabbit 10: Left side, below old lesion, edema and reddening 2 × 1.8 cm. On site of old lesion, slight edema and reddening, 0.6 × 0.5 cm.

To the side of old lesion (approximately 5 cm. distant) marked edema and reddening 2.3 cm. in diameter.

Right side, marked edema and reddening 3.5 × 4 cm. (Figs. 5 and 6).

Rabbit 11: Left side, below old lesion, slight edema and very slight reddening 1 × 1.5 cm.

On site of old lesion, slight edema, no reddening, 0.8 × 1.3 cm.

To the side of old lesion, edema and reddening 1.8 × 2 cm.

Right side, reddening and marked edema 2 × 2.3 cm.

The point of greatest resistance to a subsequent infection is shown to be the site of the old lesion; an area ventral to the old lesion is more resistant than one the same distance away on the side or dorsal to it. The uninoculated side remains susceptible.

Local Skin Resistance Induced by Heterologous Erysipelas Strain.

Thus far, the homologous strains have been used in testing the relative resistance to subsequent injections. To determine the effect of injections of a heterologous strain, rabbits were immunized against Strain B and others against Strain P, both representative beta hemolytic streptococci from cases of human erysipelas but differing immunologically as shown by their failure to cross-agglutinate.

When the rabbits had become locally immune against the homologous strain, endodermal injections of both strains were made into each rabbit and into a normal control.

Sample protocols are given below.

Experiment 6.

Rabbit 12.—Immunized against Strain B. Injections started Oct. 3, 1925, and repeated once a week for 9 weeks; after lapse of 2 months, recommenced Feb. 4, 1926, and continued until Apr. 7.

Apr. 7. Injected endodermally with 0.2 cc. of broth containing 1/10 of the 18 hour growth of Strain B on a rabbit blood agar slant, and with an equal dose of Strain P. Resulting lesion, Strain B: erythema 2 cm. in diameter with small central nodule 0.8 cm. in diameter; Strain P: erythema 3 cm. in diameter, central nodule 0.4 cm. (Fig. 7).

Rabbit 3.—Immunized against Strain P over same period as Rabbit 12.

Apr. 7, 1926. Injected endodermally with Strains B and P in same doses as Rabbit 12. Resulting lesions, Strain B, erythema 2.5 cm. in diameter, slight induration; Strain P, slight erythema and induration 1 cm. in diameter (Fig. 8).

Rabbit 13.—No previous injections. Control for Rabbits 12 and 3.

Apr. 7, 1926. Injected endodermally with Strains B and P in same doses as Rabbits 12 and 3. Both resulting lesions 2 × 10 cm. indurated and reddened with small patches of skin necrosis at points of inoculation (Fig. 9).

Rabbit 14.—June 5, 1926. Injected endodermally on right side 0.2 cc. of broth containing 1/500 of the 18 hour growth of Strain B on a rabbit blood agar slant. Resulting lesion 2.8 × 1.7 cm., edema and reddening with small patch of skin necrosis. There was also a generalized erythema.

July 3. Injected endodermally with 0.1 cc. of broth containing 1/500 of the 18 hour growth of Strain C on a rabbit blood agar slant. Injections made at the following places: (1) on old lesion; (2) below old lesion; (3) on same side, 3 cm. away; (4) on the other side.

Resulting lesions, (1) on old lesion, very small hard red nodule 0.3 cm. in diameter; (2) below old lesion, markedly red and edematous, 1.5 × 0.7 cm.; (3) to the side of old lesion, reddening and edema 1.5 × 1.9 cm., with very small patch of skin necrosis; (4) on the other side, reddening and edema 1.5 × 1.6 cm. with very small patch of skin necrosis.

Rabbit 15.—June 5, 1926. Injected endodermally on the right side with 0.2 cc. containing 1/500 of the 18 hour growth of Strain P on a rabbit blood agar slant. Resulting lesion, slight edema, 1.8 × 6.2 cm.; practically no reddening; extensive skin necrosis, 1 × 3 cm.

July 3. Injected endodermally 0.1 cc. of broth containing 1/500 of the 18 hour growth of Strain B on a rabbit blood agar slant. Injections made at the following places: (1) on site of old lesion; (2) below old lesion; (3) on same side, 3 cm. away; (4) on the other side.

Resulting lesions, (1) on old site, very small hard red papule, 0.3 cm. in diameter; (2) below old lesion, barely palpable, faint reddening 1 cm. in diameter; (3) to the side of old lesion, barely palpable slight reddening 1.5 cm. in diameter; (4) on the other side, distinct edema and reddening 1.5 × 2 cm.

Rabbit 16.—June 5, 1926. Injected endodermally on the right side 0.2 cc. of broth containing 1/500 of the growth of Strain C on a rabbit blood agar slant. Resulting lesion 2.5 × 2.8 cm., marked edema and reddening.

July 3. Injected endodermally 0.1 cc. of broth containing 1/500 of the 18 hour growth of Strain B on a rabbit blood agar slant. Injections made at the following places: (1) on site of old lesion; (2) below old lesion; (3) on same side, 3 cm. away; (4) on the other side.

Resulting lesions, (1) on old lesion, faint reddening and very slight edema 1 × 1.5 cm.; (2) below old lesion, faint reddening and very slight edema 1.8 cm. in diameter; (3) to the side of old lesion, edema barely palpable, reddening very faint 0.8 cm. in diameter; (4) on the other side, slight reddening and very slight edema 1 × 1.7 cm.

The experiments show very definitely that one member of the erysipelas group of streptococci is capable of stimulating local skin resistance against a second member of the group which is immunologically different from the first. This offers some hope of attaining a polyvalent anti-erysipelas serum which is so desirable and attests to

the close relation of members of this group even though their aggluti-
nation reactions do not establish a complete identity.

Local Resistance to Other Hemolytic Streptococci.

Rabbits immunized to Erysipelas Strains B, P, and H were tested
against a strain of beta hemolytic streptococcus isolated from the
tonsils during the acute stage of follicular tonsillitis.

The test tonsillitis strain, Heyder, was passed through three rabbits
to insure constant virulence and to ascertain the infecting dose in the
normal rabbit skin.

The results of this experiment are shown in the following protocols.

Experiment 7.

Rabbit 12.—Immunized against Strain B.   Injections started Oct. 3, 1925, and
repeated once a week for 9 weeks; after lapse of 2 months, recommenced Feb. 4,
1926, and continued until June 2.

June 2.   Injected endodermally with 0.1 cc. of broth containing 1/20 of the 18
hour growth of Strain B on a rabbit blood agar slant, and with 1/10 of the 18 hour
growth of the Heyder strain on a rabbit blood agar slant.   Resulting lesions after
48 hours, Strain B, faint reddening 0.9 cm. in diameter, small elevation 0.3 cm. in
diameter; Heyder strain, moderate edema and reddening 3 × 3.2 cm.

Rabbit 3.—Immunized against Strain P over same period as Rabbit 12.

June 2, 1926.   Injected endodermally with Strain B and the Heyder strain in
same doses as Rabbit 12.   Resulting lesions, Strain B, reddening and edema 2.1 ×
1.3 cm.; tonsillitis strain, edema and very slight reddening 2 × 2.5 cm.

Rabbit 6.—Immunized against Strain H.   First injection Sept. 28, 1925, second,
Nov. 11, repeated once a week until Dec. 27.   Recommenced Jan. 29, 1926, and
continued until June 2.

June 2.   Injected endodermally with Strain B and the Heyder strain in same
doses as Rabbits 12 and 3.   Resulting lesions, Strain B, slight edema and redden-
ing 1.2 × 1.8 cm.; Heyder strain, edema and reddening, 3.2 × 1.5 cm.

Rabbit 17.—No previous injections.   Control for Rabbits 12, 3, and 6, in this
experiment.

June 2, 1926.   Injected endodermally with Strain B and the Heyder strain in
same doses as Rabbits 12, 3, and 6.   Resulting lesions, Strain B, edema and
reddening, 2.0 × 1.8 cm., with large patch of skin necrosis; Heyder strain, slight
edema and reddening, 1.5 × 4.5 cm., with small patch of skin necrosis.

Experiment 8.

Rabbit 18.—June 2, 1926.   Injected endodermally on right side 0.1 cc. of broth
containing 1/500 of the 18 hour growth of Strain B on a rabbit blood agar slant.

Resulting lesion, 1.8 × 2.3 cm., reddening and edema with small central patch of skin necrosis.

July 2. Injected endodermally 0.1 cc. of broth containing 1/20 of the growth of the Heyder strain on a rabbit blood agar slant 24 hours old. Injections made at the following places: (1) on old lesion; (2) below it; (3) on the same side, 3 cm. away; (4) on the other side.

Resulting lesions, (1) on old lesion, small red papule 0.5 × 0.8 cm.; (2) below old lesion, slight reddening and edema 1.5 × 1.8 cm.; (3) to the side of old lesion, reddening and edema 1 cm. in diameter; (4) on the other side edema and reddening 1.5 × 3.5 cm. with a purplish hemorrhagic centre (Figs. 10 and 11).

These tests show that the skin of a rabbit immunized against a strain of beta hemolytic streptococcus from erysipelas possesses considerable resistance to beta hemolytic streptococci from another source, but less than that exhibited against the erysipelas strain.

Local Resistance to Staphylococcus aureus.

Rabbits immunized to Erysipelas Strains B, P, and H were given endodermal injections of test doses of a culture of *Staphylococcus aureus* isolated from a human case of staphylococcemia following cellulitis. The test strain had been passed through five rabbits and was found to produce a marked reaction when injected into the skin of the normal rabbit. The results of these tests are shown in the following protocol.

Experiment 9.

Rabbit 12.—Immunized against Strain B. Injections started Oct. 3, 1925, and repeated once a week for 9 weeks; after lapse of 2 months, recommenced Feb. 4, 1926, and continued until June 2.

June 2. Injected endodermally 0.1 cc. of broth containing 1/20 of the 18 hour growth of Strain B on a rabbit blood agar slant and with 1/20 of the 18 hour growth of a *Staphylococcus aureus* strain on a 7.4 beef infusion agar slant. Resulting lesions after 48 hours; Strain B, faint reddening 0.9 cm. in diameter, small elevation 0.3 cm. in diameter; staphylococcus strain, after 48 hours, slight reddening 1 cm. in diameter, small yellowish elevation 0.3 cm. in diameter.

Rabbit 3.—Immunized against Strain P over same period as Rabbit 12.

June 2, 1926. Injected endodermally with Strain B and the staphylococcus strain in same doses as Rabbit 12. Resulting lesions, Strain B, reddening and edema 2.1 × 1.3 cm.; staphylococcus strain, reddening and edema 1.8 × 0.8 cm.

Rabbit 6.—Immunized against Strain H. First injection Sept. 28, 1925, second on Nov. 11, repeated once a week until Dec. 27. Recommenced Jan. 29, 1926, and continued until June 2.

June 2. Injected endodermally with Strain B and the staphylococcus strain in same doses as Rabbits 12 and 3. Resulting lesions, Strain B, slight edema and reddening 1.2 × 1.8 cm.; staphylococcus strain, edema and reddening 1.3 × 1.8 cm.

Rabbit 17.—No previous injections. Control for Rabbits 12, 3, and 6 in this experiment.

June 2. Injected endodermally with Strain B and the staphylococcus strain in same doses as Rabbits 12, 3, and 6. Resulting lesions, Strain B, edema and reddening, 2.0 × 1.8 cm., with large patch of skin necrosis; staphylococcus strain, edema and reddening, 2 cm. in diameter, with small patch of skin necrosis.

It appears from these experiments that the skin of rabbits immunized against beta hemolytic streptococci from erysipelas is also slightly resistant to infection by *Staphylococcus aureus*.

Resistance to Staphylococci of Contiguous Areas.

To test the spread of local resistance to staphylococci from an area previously injected with streptococci, an endodermal injection of Hemolytic Streptococcus C was made on the left side of a rabbit and after an interval of 30 days the contiguous areas were inoculated with the staphylococcus. Details of the experiment are shown in the following protocol.

Experiment 10.

Rabbit 19.—June 2, 1926. Injected endodermally on right side with 0.1 cc. of broth containing 1/500 of the 18 hour growth of Strain C on a rabbit blood agar slant. Resulting lesion, reddening and marked edema, 1.7 × 1.8 cm., with narrow zone of hemorrhage round the edge.

July 2. Injected endodermally 0.1 cc. of broth containing 1/20 of the growth on a 7.4 beef infusion agar slant of the virulent strain of *Staphylococcus aureus*. Injections made at the following places: (1) on old lesion; (2) below old lesion; (3) on the same side, 3 cm. away; (4) on the other side (Figs. 12 and 13).

Resulting lesions, (1) on the old lesion, reddening and marked edema, 0.8 × 1.6 cm., with tiny patch of skin necrosis; (2) below old lesion, reddening and marked edema, 1.8 × 2.8 cm., with small patch of skin necrosis; (3) to the side of old lesion, reddening and marked edema, 2.3 × 3.5 cm., with small patch of skin necrosis; (4) on the other side, reddening and marked edema, 2 × 2.3 cm., with small patch of skin necrosis.

Thus, 30 days after an injection of streptococci, the area of skin involved in the inflammatory reaction was somewhat refractory to an injection of virulent staphylococci. But the difference between the

lesion on the treated and that produced on the other side of the rabbit was not so great as in the experiments in which the same strain of streptococci was employed both for the local immunizing inoculation and the subsequent tests.

As in the experiment in which the same strain of streptococci was used the area ventral to the site of previous injection was refractory to subsequent injection but the non-specific resistance was not as great as the specific resistance. The endodermal injection of staphylococci anterior to the area involved in the immunizing process resulted in a lesion somewhat larger than that on the other side of the rabbit where no previous injection had been made. This result is in accordance with the experiment in which the homologous strain was used to test the direction of the spread of the local immune area.

Repeated endodermal injections induce a gradual spread in the areas of increased resistance until general immunity is established. After the lesion produced by the original inoculation has subsided, usually in 2 or 3 weeks, the skin which had been involved is highly resistant to subsequent infection. The contiguous areas are slightly resistant and the regions in the assumed direction of the lymph flow still more refractory to subsequent infection. At this time the skin on the other side of the rabbit has not acquired any resistance. As the immunization proceeds by weekly injections on one side, the opposite side which has never been injected begins to show resistance. But, at the same time, there is evidence of humoral immunity as shown by the presence of agglutinins and antitoxic properties in the blood serum.

There are differences not shown in the averages. As the weekly injections were made, the redness and edema diminished but no records were kept of the extent to which the lesions were raised above the surrounding tissues. There are, of course, exceptions to the averages, for example, in one instance the lesion after the first injection measured 3 cm. and after the second, 5 cm., yet after the third and fourth, 0.6 and 0.2 cm. respectively.

In the course of repeated injections when the lesions produced by the standard dose decreased progressively in size, the reaction within an hour after injection was sometimes surprisingly large and was characterized by great edema rather than redness, and by abrupt subsidence. The succeeding injections did not regularly show this

phenomenon. The same observations have been made in horses that were undergoing immunization by the endodermal method. The question of an allergic response will be discussed in later publications.

These results suggest the gradual development of skin resistance to repeated endodermal inoculation.

SUMMARY.

The experiments recorded show that the skin involved in the zone of inflammation produced by the endodermal injection of virulent living erysipelas streptococci into rabbits becomes, after the lesion has healed, partially but not completely resistant to subsequent infection with the homologous organism. In the majority of experiments the lesion resulting from the first endodermal injection spread downward, *i.e.*, ventrally, to the site of needle puncture forming an oval or elongated inflammatory zone. Subsequent injections of the same strain into the skin beyond and ventral to the apparent edge of the lesion showed that these areas in the supposed direction of the lymph flow likewise became resistant but not quite to the same degree as in the inflammatory area. The contiguous areas above, behind, and in front of the healed lesion exhibited only a very mild degree of resistance. The other side of the rabbit where no previous injections had been made reacted as did the normal skin.

Repeated injections on the same side bring about diminishing local reaction until there is almost no lesion following the injection of the standard skin dose. Thus there is a gradual spread of skin resistance on the inoculated side, whereas the non-injected side of the rabbit reacts normally. Finally, however, after many injections over relatively long periods the non-injected side becomes resistant, but at this time, there is evidence of general humoral immunity as shown by the presence of agglutinins and antitoxin in the blood. The local resistance is apparently not entirely specific, for the areas with the previous lesion and ventral to it become more resistant to another strain of beta hemolytic streptococcus, though in less degree, and to a virulent strain of *Staphylococcus aureus* to a still less degree. The most plausible explanation of the spread of the local immunity ventrally is that the streptococci follow the lymph channels. Indeed, in human

erysipelas, the organisms are recovered by cutipuncture as far as 3 cm. beyond the advancing edge of the lesion where there is no gross evidence of inflammation. This aspect of local immunity will be considered in subsequent publications.

The skin of the rabbit involved in the inflammatory reaction following the endodermal injection of living streptococci becomes resistant to subsequent injection of the homologous strain and of other strains of erysipelas streptococci which are not immunologically identical.

The local immune areas are resistant but not to the same degree to a strain of hemolytic streptococci isolated from follicular tonsillitis and to a virulent *Staphylococcus aureus*.

The areas contiguous to the local lesion but outside the apparent boundary of inflammation become more resistant to subsequent injection. But the regions ventral to those areas become more resistant than those dorsal, anterior, or posterior to the inflammatory zone.

The skin on the non-injected side of the rabbit becomes resistant *pari passu* with the development of humoral immunity.

EXPLANATION OF PLATES.

PLATE 8.

FIG. 1. Rabbit 2—Experiment 3. Lesions on left side resulting from injections below site of a previous inoculation and adjacent to it. The white spot at the lower edge of shaved area and below a point of inoculation is due to complete depilation and not edema.

FIG. 2. Same rabbit. Lesion on right side which had received no previous injection.

FIG. 3. Rabbit 9—Experiment 5. Lesions on left side resulting from injections on the site of an old lesion, below it, and adjacent to it.

FIG. 4. Same rabbit. Lesion on right side which had received no previous injection.

FIG. 5. Rabbit 10—Experiment 5. Lesions on left side resulting from injections on the site of an old lesion (*b*), below it (*c*), and anterior to it (*d*). The point of original inoculation is *a*.

FIG. 6. Same rabbit. Lesion on right side which had received no previous injection.

PLATE 9.

FIG. 7. Rabbit 12—Experiment 6. Immunized against Strain B. Lesion at left produced by Strain B; that at right, by Strain P.

FIG. 8. Rabbit 3. Immunized against Strain P. Lesion at left produced by Strain B; that at right, by Strain P.

FIG. 9. Rabbit 13. No previous injections. Control for Rabbits 12 and 3 in above experiment.

PLATE 10.

FIG. 10. Rabbit 18—Experiment 8. Lesions on right side resulting from injections with a tonsillitis strain on the site of a previous inoculation with an erysipelas strain, below the site and posterior to it.

FIG. 11. Same rabbit. Left side which had received no previous injection.

FIG. 12. Rabbit 19—Experiment 10. Lesions on right side resulting from injections with a strain of *Staphylococcus aureus* on, below, and posterior to the site of a previous inoculation with an erysipelas strain.

FIG. 13. Same rabbit. Left side which had received no previous injection.

(Amoss and Bliss: Local immunity in experimental erysipelas.)

BLOOD FIBRIN IN UPPER GASTROINTESTINAL TRACT OBSTRUCTION.

By RUSSELL L. HADEN, M.D., AND THOMAS G. ORR, M.D.

(*From the University of Kansas School of Medicine, Kansas City, Kansas.*)

Foster and Whipple (1–4) have studied the blood fibrin in a number of experimental conditions. They have shown that fibrin is a very labile protein with wide fluctuations due to a variety of stimuli. The site of production is mainly the liver where there is a constant manufacture to supply the continuous utilization in the animal body. Injury to any tissue except the liver causes some increase in the fibrin content of the blood. If the liver is seriously injured a decrease is observed. With mild liver injury there is a rise instead of a fall.

Dead or injured tissue and non-specific inflammation seem the only factors needed to excite an increased production. It seems quite probable also that any cell injury even without inflammatory reaction will cause this characteristic rise. McLester (5) has attempted to utilize blood fibrin determinations as an index of liver function. Foster (6) made fibrin determinations in numerous clinical conditions. He found a marked elevation with the toxemia of pregnancy, the fibrin level running parallel with the severity of the symptoms. With nephritis no increase was observed. Liver atrophy showed a very low value and lobar pneumonia a very high value.

We have made a series of plasma fibrin determinations for comparison with data previously observed in the toxemia incident to upper gastrointestinal tract obstruction.

Methods.

All experiments were done on dogs. All operations were done under anesthesia with aseptic technique. The cardiac end of the stomach and pylorus were obstructed by ligation with tape. The jejunum was obstructed by severing the intestine and turning in the cut ends. The hematocrit reading was obtained by centrifuging 10 cc. of blood mixed with 2 cc. of 1.6 per cent sodium oxalate. The

fibrin determinations were made on this oxalated plasma by the method of Foster and Whipple (1). The non-protein nitrogen was determined by the method of Folin and Wu (7) and the chloride after the manner suggested by Gettler (8).

TABLE I.

Obstruction of Cardiac End of Stomach.

Dog No.	Day after operation	Hematocrit reading (per cent of cells)	Fibrin (mg. per 100 cc. plasma)	Blood (mg. per 100 cc.)	
				Non-protein nitrogen	Chlorides (as NaCl)
		per cent	*mg.*	*mg.*	*mg.*
11	0	55	605	47.6	530
	1	51	980	46.0	490
	2	51	1350	65.5	480
	3	54	1717	101.0	470
	4	43	1608	106.0	570
4	0	50	569	44.4	520
	1	56	1020	60.0	470
	2	61	1210	45.6	440
	3	52	1468	54.5	420
	4	50	1554	118.0	440
	5	55	1600	163.0	490
7	0	50	572	50.5	520
	1	63	972	28.9	490
	2	63	1006	78.0	500
	3	64	1414	130.0	520
8	0	58	665	28.9	470
	1	64	1170	31.9	450
	2	67	1679	82.5	440
	3	61	1828	139.0	400
12	0	54	520	37.3	480
	1	62	975	140.0	410
	2	60	1122	152.0	380

EXPERIMENTAL OBSERVATIONS.

Successive determinations of plasma fibrin after obstruction of the cardiac end of the stomach are shown in Table I. In each animal there is a marked increase, the fibrin being almost tripled. The fibrin

increase parallels quite closely the degree of toxemia as indicated by the level of the non-protein nitrogen of the blood.

TABLE II.

Obstruction of Pylorus.

Dog No.	Day after operation	Hematocrit reading (per cent of cells)	Fibrin (mg. per 100 cc. plasma)	Blood (mg. per 100 cc.)		Remarks
				Non-protein nitrogen	Chlorides (as NaCl)	
		per cent	*mg.*	*mg.*	*mg.*	
13	0	53	368	32.6	480	
	2	61	564	67.5	460	
	3	53	1721	139.0	440	
14	0	46	388	38.9	390	
	1	53	926	36.6	—	
	2	54	840	48.7	450	
	4	61	1212	167.0	420	
10	0	45	566	33.0	470	
	2	60	1380	68.3	420	Obstruction released
	4	48	1301	113.0	320	
	5	42	1148	91.0	440	
15	0	53	456	59.7	490	
	1	57	513	75.0	400	
	2	60	1326	70.0	360	" "
	3	50	1348	107.0	330	
9	0	66	324	30.0	490	
	1	64	1109	30.3	420	
	2	64	1241	59.5	330	" "
	3	58	1036	41.6	390	
	4	58	1062	41.0	380	
	5	69	766	32.6	380	
	6	57	756	38.9	390	
	7	45	751	31.9	400	
	8	43	719	27.0	400	
	9	40	677	35.3	390	
	10	42	845	32.6	420	

With obstructions of the pylorus (Table II) the findings are quite similar. The increase here is even more marked and rapid. In three

animals the obstruction was released after onset of the toxemia. In two the fibrin level remained high and the animals died. One recovered. In this animal the fibrin fell but did not return to the normal level. In four dogs with the jejunum obstructed there was also an increase in the fibrin level. This was not so marked however as with

TABLE III.

Obstruction of Jejunum.

Dog No.	Day after operation	Hematocrit reading (per cent of cells)	Fibrin (mg. per 100 cc. plasma)	Blood (mg. per 100 cc.)	
				Non-protein nitrogen	Chlorides (as NaCl)
		per cent	*mg.*	*mg.*	*mg.*
16	0	44	686	26.5	460
	1	51	1238	33.0	460
	2	54	1358	116.0	390
17	0	47	489		430
	1	47	820	25.7	390
	2	49	1000	31.9	300
	3	46	681	65.0	250
18	0	50	670	46.8	450
	1	65	801	86.3	360
19	0	56	620	34.5	440
	1	60	813	41.4	350
	2	58	953	63.0	320
	3	58	905	12.2	320
	4	53	760	105.0	270
	6	55	650	126.0	220
	8	55	692	179.0	200
	10	53	725	234.0	180

obstruction of the cardia and pylorus. One animal (No. 19) showed an exceedingly low blood chloride and high non-protein nitrogen with no marked change in the fibrin.

DISCUSSION.

The very high blood fibrin level observed in this series of animals emphasizes the tissue injury occurring in such conditions. The rise

is especially striking after obstruction of the cardiac end of the stomach which we have shown is characterized by a toxemia more serious than that incident to pyloric or high intestinal obstruction.

These findings are of interest also in indicating that hepatic insufficiency is not the cause of death in high gastrointestinal tract obstruction as has been suggested (9).   If there is any liver injury it must be mild since extensive liver injury without exception has been shown to give low values for fibrin.

<div style="text-align:center">SUMMARY.</div>

After high gastrointestinal tract obstruction the blood fibrin increases rapidly.

The change is more marked in animals with obstruction of the cardiac end of the stomach.

The rise in fibrin parallels closely the toxemia characteristic of such conditions.

These results indicate that liver insufficiency cannot be the cause of death in such obstructions. .

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THE EXCRETION OF NITROGEN AFTER UPPER GASTRO-INTESTINAL TRACT OBSTRUCTION.

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A high level of non-protein nitrogen in the blood is characteristic of the toxemia incident to upper gastrointestinal tract obstruction (1, 2). The explanation for this change is not entirely clear. Whipple, Cooke and Stearns found an increase in nitrogen excretion in the dog after intestinal obstruction (3), and no marked evidence of renal insufficiency (4). They attributed the heaping up of non-protein nitrogen in the blood to accelerated protein destruction caused by some toxic body arising in the wall of the obstructed gut. More recently there has been a tendency to doubt the existence of a toxic body and to consider the increase in the non-protein nitrogen of the blood as a true retention due entirely to renal insufficiency, possibly caused in turn by dehydration. Convincing proof for this view is lacking. The high blood urea concentration observed in children suffering from water loss produced by gastrointestinal tract disturbances, has also been interpreted as due to renal insufficiency without any adequate proof (5). Gamble and Ross (6) in studying experimentally the dehydration of rabbits, after pyloric obstruction, make a similar assumption.

The question can be settled only by determining accurately the nitrogen excretion. In experimental pyloric and intestinal obstruction this is most difficult, since collections of urine can seldom be accurately made on account of vomiting. We have shown that the toxemia incident to obstruction of the cardiac end of the stomach is similar to but more marked than that following high intestinal or pyloric obstruction (7). Since here no vomiting is possible, the urine can be quantitatively collected. We have also found that if the pylorus is obstructed for 48 hours and then released, although the vomiting

ceases, making urine collections possible, the toxemia often continues. We have determined the non-protein nitrogen excretion in six dogs with obstruction of the cardia, in two after obstruction and release of the pylorus and in two control dogs.

TABLE I.

Excretion of Nitrogen after Upper Gastrointestinal Tract Obstruction.

Dog	Dura-tion of experi-ment	Blood (mg. per 100 cc.)				Urine (per kilo of body weight per 24 hrs.)		Operation
		Beginning		End		Amount	Total non-protein nitrogen	
		Urea nitro-gen	Non-protein nitro-gen	Urea nitro-gen	Non-protein nitro-gen			
	days	mg.	mg.	mg.	mg.	cc.	gm.	
1	4	....	....	....	....	13.0	0.141	None (control)
2	5	....	....	....	....	5.5	0.122	" "
				Average		9.3	0.132	
3	3	16.6	26.0	58.8	167.0	29.0	0.391	Obstruction of cardia
4	4	9.8	44.4	66.5	116.0	19.7	0.248	" " "
5	2	16.1	33.0	30.1	59.0	19.8	0.217	" " "
6	3	12.6	27.7	27.9	51.8	24.5	0.337	" " "
7	3	25.2	50.5	30 1	78.0	23.3	0.230	" " "
8	3	9.8	28.9	46.8	82.5	19.8	0.266	" " "
				Average		22.7	0.289	
9	7	38.5	59 5	11.9	35.3	41.8	0.557	Pylorus obstructed for 48 hrs. and released
10	10	98.1	132.0	14.7	25.2	25.0	0.413	" "
				Average		33.4	0.485	

The animals were kept in metabolism cages. All operations were done under anesthesia with aseptic technique. No food was given but water was allowed *ad libitum*. The non-protein nitrogen in the urine was determined by a micro Kjeldahl method (8), the urea nitrogen of the blood by the Van Slyke and Cullen modification of the Marshall method (9), and the non-protein nitrogen by the Folin and Wu method 10).

EXPERIMENTAL OBSERVATION.

The blood and urine findings are shown in Table I. All figures have been reduced to kilo of body weight per 24 hour period. The average excretion of non-protein nitrogen in the control animals per kilo for the 24 hour period is 0.132 gm. and the fluid excretion 9.3 cc. After cardiac obstruction the average of six dogs is 0.289 gm., an increase of 119 per cent. The water excretion is also increased from 9.3 cc. to 22.7 cc. per kilo per 24 hour period. The non-protein nitrogen excretion in two dogs, after obstruction and release of the pylorus, was 0.485 gm., an increase of 267 per cent, and water excretion 33.4 cc. per kilo per 24 hour period.

DISCUSSION.

In both cardiac obstruction and after obstruction and release of the pylorus there is a very marked increase in non-protein nitrogen and water excretion, as compared with the controls. With the increase in nitrogen excretion in the animals with cardiac obstruction there is also an increase in the non-protein nitrogen of the blood. These findings could only be explained by a marked increase in protein destruction. Dog 4 showed an increase in urea nitrogen from 9.8 mg. to 66.5 mg. per 100 cc. of blood. It has been shown that for every gm. of nitrogen retained as urea in a man weighing 70 kilos there is an increase of 1.33 mg. of urea per 100 cc. of blood (11). If these figures are applied to this dog the increase in urea nitrogen in the blood would in addition correspond to an excretion of 0.314 gm. of nitrogen per kilo per 24 hour period as urea alone. Mackay and Mackay (12) have shown that in fasting in rabbits there is a marked increase in protein destruction. This they consider secondary to the marked water loss since the increase is not due to kidney retention. Our animals also had a marked water loss. Whether the increased protein destruction is due to this cause or some toxic body is not determined. The nitrogen excretion, after pyloric obstruction and release of the pylorus, is high. The urea nitrogen and non-protein nitrogen of the blood was decreasing and some nitrogen was thus washed out. This could account for only a fraction of the nitrogen excreted, however.

SUMMARY AND CONCLUSION.

After obstruction of the cardiac end of the stomach and after obstruction and release of the pylorus there is a marked increase in non-protein nitrogen excretion.

This increase is due to accelerated protein destruction, which may result from the great fluid loss, or to the action of some toxic body.

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ETIOLOGY OF OROYA FEVER.

VI. Pathological Changes Observed in Animals Experimentally Infected with Bartonella bacilliformis.

The Distribution of the Parasites in the Tissues.

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PLATES 11 TO 14.

Experimental infection with *Bartonella bacilliformis* in young *Macacus rhesus* monkeys varies in severity according to the susceptibility of the individual animals[1,2,3] and is of three general types: (1) severe, fatal affection characterized by extreme anemia, similar to Oroya fever in man, (2) relative benign condition, in which there is moderate anemia or none at all, accompanied by localized skin lesions resembling those of human verruga, (3) mild systemic disease, involving the lymphatic glands and blood.

The pathological changes found in human organs and tissues after death from *Bartonella bacilliformis* infection have been described by Odriozola,[4] Arce,[5] Hercelles,[6] and by Strong and his coworkers.[7] The characteristic effects are seen in the liver, spleen, lymphatic glands, and bone marrow. Hypertrophy of the liver is almost invariable,[4,5] and the spleen is frequently enlarged,[4,7] though it may

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be of normal size or less.[5] Infarcts are commonly found in the spleen and sometimes also in the liver.[6, 7] Strong and his coworkers note particularly the areas of degeneration in the liver, beginning about the hepatic veins, apparently due to the activity of the parasite and suggesting the presence of a toxin in the circulating blood. The lymphatic glands are enlarged everywhere[4, 5, 7] and frequently edematous. The hypertrophy is most marked in the mesenteric lymph nodes.[4] The marrow of the long bones is red or mottled with red patches,[4, 7] and distinctly softer than normal. Microscopically there is evidence of phagocytosis by endothelial leucocytes of red cells and polymorphonuclear leucocytes, also increased production of normoblasts.[7] Extensive phagocytosis by endothelial cells is found also to occur in the spleen, while the lymphatic glands often contain many large swollen endothelial cells in which the bacilliform parasites are present in large numbers.[7] Similar intracellular parasites may be found[7] in the blood vessels near ulcerated areas in the intestinal wall. The parasite has a marked predilection for the reticulo-endothelial system and sets up an active and persistent proliferation of endothelial cells in the tissues or organs which it invades.

The characteristic nodular lesions caused by *Bartonella bacilliformis* have occasionally been found to occur in the muscles and viscera as well as on the mucous membranes.[4, 5, 8, 9] Campodónico, in 1895,[10] reported the finding at autopsy, in a young child who succumbed to severe anemia during the course of verruga, of tiny red nodules in all the tissues of the body.

In the early experimental work with *Bartonella bacilliformis* only the milder types of infection were observed, and it was not until the third and fourth generations in monkeys that it was possible to induce, by massive inoculations of passage strains, a fatal disease accompanied by extreme anemia comparable with Oroya fever of man. Five animals succumbed to the infection, and, as in the case of the human disease, the conspicuous changes were found in the liver, spleen, bone marrow, and lymphatic glands. It was desirable, therefore, not only to compare the findings with those occurring in human material, but also to relate the histological changes in a particular tissue with the presence or absence of the parasite. In order better to accomplish this purpose a number of infected ¦animals were sacrificed either at the height of disease, as evidenced by extensive local lesions and a high titer of the parasite in the blood, or during the period of convales-

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cence, when the blood findings were negative and the local lesions had subsided.

The important fact should be emphasized that tuberculosis was not encountered among the animals of the present series, notwithstanding special search was made for *B. tuberculosis* because of the possibility of confusion from this source.

Although microscopic examination of all materials—blood, organs, or tissues—was always made, the cultural method was found to be more reliable for determining the presence of *Bartonella bacilliformis*. Moreover, secondary infection is readily detected by this method. About 0.1 cc. of each of several dilutions (from 1:10 up to 1:1,000,000) of blood or saline suspension of tissue is introduced into leptospira medium, and the tubes are kept constantly at 25°C. for 4 to 5 weeks and examined periodically. Growth is often detected only after 2 to 3 weeks.

Films were always made of blood and impression smears of spleen, bone marrow, lymph glands, and liver. They were dried in air, fixed in methyl alcohol for 5 to 10 minutes, and stained with Giemsa's solution for 30 to 60 minutes.

Tissues were fixed in Regaud's fluid, and the sections stained with hematoxylin and eosin for histological study and with Giemsa's solution for detection of *Bartonella bacilliformis*.

The fourteen monkeys (*Macacus rhesus*) serving as material for the present study will be considered in three groups: (1) five animals which died of experimental infection with *Bartonella bacilliformis;* (2) five animals which showed signs of infection, locally and constitutionally, when sacrificed; (3) four animals which had shown signs of infection but were apparently in convalescence when sacrificed.

In the first group, three showed marked and two extreme anemia during the latter part of the disease or at the time of death. In Monkey 25 the red blood cells numbered 1,164,000 per c.mm. and the hemoglobin (Sahli) was 25 per cent; in Monkey 37 the red cells numbered 1,176,000 and the hemoglobin was 15 per cent. In these animals a terminal secondary bacterial infection intervened 12 and 96 hours before death.

There was moderate anemia in some of the animals of Group 2 but none in those of Group 3.

In Group 1 (fatal cases) the clinical picture was that of Oroya fever, and the pathological changes likewise proved to be similar to those

observed in the human infection.   In Group 2 (non-fatal cases), the manifestations were characteristic of human verruga.

Pathological Findings in Fatal Infections with Bartonella bacilliformis.

The following outline is a composite picture of the characteristic changes noted.   The findings in individual instances and the relation of the cultural to the pathological findings are recorded in Table I and in the protocols.

The duration of illness was 56 days in Monkey 17, 36 days in Monkey 25, 25 days in Monkey 30, 29 days in Monkey 35, and 41 days in Monkey 37.

External Appearances.

The skin and mucous membranes have a striking waxy yellowish appearance, and the numerous swollen lymphatic glands in the axillary and inguinal regions stand out prominently beneath the stretched skin.   Pale nodules are found at the original sites of inoculation on eyebrows and abdominal skin.   In an exceptional instance (Monkey 25) there were also numerous spontaneously occurring nodules on various parts of the body.

Thorax.

Lungs.—Pale yellow, normally crepitant, smooth.   Congestion or edema at base in some instances.   Bronchial lymph nodes greatly enlarged.   Pleuræ anemic; small amount of effusion present.

Heart.—Myocardium flabby, pale, contains partially fluid pale red blood and occasionally a small clot.   No petechiæ present.   The pericardium contains some clear fluid.   In one instance (Monkey 25) an acute pericarditis due to secondary invasion was present.

Abdomen.

Liver.—Pale, flabby in consistency, frequently somewhat enlarged, often shows yellowish mottling.   Areas of necrosis and fatty degeneration are often recognizable.

Spleen.—Dark bluish red, enlarged, rather firm in consistency.   Follicles indistinct.   Scattered minute grayish red areas (infarctions) are present in all instances in varying numbers.

Kidneys.—Pale, with yellowish gray tint.

Adrenals.—Pale yellow.

Pancreas.—Normal in appearance.

Stomach.—Usually empty, mucosa pale.

Small Intestine.—Pale, serosa studded with numerous dark bluish swollen lymph nodes.   Mucosa pale.   Peyer's patches slightly hypertrophied.

Large Intestine.—Similar in appearance to small intestine, swollen lymph nodes on serosa giving it a striking aspect. No ulceration of the mucosa.

Bladder.—Normal in appearance.

Ovaries, Uterus, Testicles.—Pale, but normal.

Bone Marrow (Femur).—Dark grayish red.

Lymphatic Glands.—Entire lymphatic system hypertrophied and congested to an extraordinary degree. In the inguinal and axillary regions two or three swollen glands sometimes unite to form an irregular mass, 2 to 3 cm. in longest diameter, pink to deep red in color, and edematous. In the abdominal cavity the swollen lymph nodes are sometimes dark bluish, sometimes bluish red, and by contrast with the pale intestinal wall present a spectacular picture. They range in size from 2 to 6 mm. and are attached so firmly to the intestinal wall that they appear like verrucous nodules.

Nervous System.—The brain and spinal cord and their coverings are anemic but otherwise appear normal.

Microscopic Pathology.

Liver.—The normal pattern of cell arrangement is more or less disturbed. The hepatic cells have lost their sharp outline, stain poorly, and are vacuolated. The cells around the hepatic veins are necrotic (Fig. 1). Here and there, on the borders of the necrotic areas, between the liver cells, are engorged macrophages, which often contain erythrocytes, polymorphonuclear leucocytes, and erythroblasts. Around the portal veins are several layers of proliferated endothelial cells. In some specimens yellowish or brownish pigment in granules or small masses is found within the endothelial phagocytes or in endothelial cells lining the sinusoids. The degree of necrotic change around the hepatic veins is variable in the different animals. In some areas the liver cells are clear and homogeneous, in others coarsely granular. A few endothelial cells containing *Bartonella* were found after prolonged search in two of the five livers (those of Monkeys 17 and 30).

Spleen.—(Figs. 7, 8, 23.) Many infarctions are found, the periphery showing invasion of leucocytes. Many of the veins are occluded, owing probably to endothelial hyperplasia. The splenic nodules are reduced in size and are often scattered through the proliferating endothelial cells. Engorged macrophages containing erythrocytes are quite abundant. Yellowish brown pigment is present, some in the endothelial leucocytes, some between the splenic cells.

A number of endothelial cells containing masses of elements resembling *Bartonella bacilliformis* were found (Figs. 16, 25), but none of the swollen endothelial cells containing rods and granules such as were seen in human materials by Strong and his coworkers.

Lymphatic Glands.—The principal changes in these glands are the increase in the number of endothelial cells and the presence of numerous engorged macrophages. In the mesenteric nodes a large amount of greenish pigment is found in the phagocytic endothelial cells of the sinuses. *Bartonella bacilliformis* has been observed in a few endothelial cells in Monkeys 29 and 30 (Fig. 27).

Bone Marrow (Femur).—Numerous macrophages containing erythrocytes, polymorphonuclear leucocytes, and cell débris are present, and there are large numbers of normoblasts in some specimens (Monkeys 17, 25, 30). Some cells, probably endothelial, containing elements resembling *Bartonella bacilliformis* have been found in film preparations (Figs. 15, 26).

The changes are strikingly like those described as occurring in tissues from fatal cases of Oroya fever, the differences being in degree rather than in kind. For example, the changes in the liver and spleen in monkeys are decidedly less pronounced than those seen in such human materials as are at my disposal for comparison, while the reaction in the lymphatic glands is in reverse degree, the more active phagocytosis being observed in monkeys. The origin of the minute necrotic areas in the spleen may be sought in the obstruction of capillary lumina by proliferating endothelial cells.

As in the skin lesions, so also in the spleen and lymph glands, the progressive and persistent proliferation of endothelial cells is usually associated with the presence of *Bartonella bacilliformis* in considerable numbers, as detected by cultures. The extensive zonal necrosis around the veins of the liver, which is less extensively invaded by the parasite, would appear, as suggested by Strong and his coworkers, to be due to the presence of a toxin in the circulating blood.

Pathological Findings in Animals Sacrificed during the Period of Active Infection.

The five animals of Group 2 all showed signs of active infection at the time when they were killed by etherization. The period from inoculation to the time of killing was 22 days in Monkey 4, 68 days in Monkey 5, 53 days in Monkey 23, 24 days in Monkey 24, and 28 days in Monkey 29. None of the animals showed severe anemia, although in Monkey 24 there was moderate diminution both in red blood cells and hemoglobin (erythrocytes 3,432,000, hemoglobin 55 per cent). *Bartonella bacilliformis* had been cultivated from the blood of Monkey 23 in a dilution of 1:100,000 16 days after inoculation, but it could not be recovered from undiluted blood 15 days later nor at any subsequent time.

The spleen and lymphatic glands were usually affected, and also the bone marrow as far as studied; the liver was less frequently in-

volved. Except in Monkey 23, of which special mention will be made later, the other organs were always normal in appearance.

The spleen was always more or less enlarged and rather firm; in one instance the surface was rather irregular (Monkey 5). In one animal (Monkey 23) there were numerous grayish brown areas of infarction, 1 to 1.5 mm. in diameter, scattered over the organ, which was a dark bluish red (Fig. 23). The lymphatic glands were in all cases swollen and congested and approached in size those of the fatally infected animals. The bone marrow was active in three animals studied (Monkeys 5, 23, and 24). The liver was affected to a similar degree in two animals (Monkeys 23 and 24); in the other three it was apparently normal. *Bartonella bacilliformis* was seen in a few of the endothelial cells in the liver of Monkey 24. The microscopical findings in the animals of Group 2 were identical with those in the fatally infected animals except that the changes were less pronounced in some respects.

An unusually interesting observation was made in the case of Monkey 23. On the pinkish surface of the lobules of the lungs were noticed a dozen pale, grayish, semitransparent, round nodules, measuring 1 to 2 mm. in diameter, firm, and sharply demarcated from the adjacent normal lung tissue (Fig. 22). Microscopically (Fig. 11), they consisted of numerous large mononuclear cells, many of them vacuolated and filled with dark granular pigment. The capillaries in this area, which were few in number, showed endothelial thickening. Several engorged macrophages and cell débris were also present. In a few of the endothelial cells *Bartonella bacilliformis* was found (Figs. 14, 24).

Pathological Changes in Animals Sacrificed during Convalescence.

Four animals were sacrificed when apparently in the course of convalescence from a mild infection. None showed fever or local lesions, though the lymphatic glands were still swollen in all instances. The erythrocyte count ranged from 5,472,000 to 6,584,000, and the hemoglobin was 80 to 90 per cent. The animals appeared to be in excellent condition when sacrificed 58 days (Monkey 7), 49 days (Monkey 8), 40 days (Monkey 10), and 30 days (Monkey 13) after inoculation.

The changes in these animals, when still evident, were characteristic. The spleen was somewhat enlarged in three instances and in two showed definite endothelial hyperplasia; in one (Monkey 13) there was a marked increase in fibrous tissue; in another (Monkey 7) the same changes were noted as had been found in the spleens of the actively infected animals. The liver was macroscopically normal in all four animals. The lymph nodes were active in two (Monkeys 7 and 8) but quiescent in the others. The bone marrow was normal in Monkeys 8, 10, and 13, and active in Monkey 7.

The findings suggested that Monkeys 7 and 8 were still infected and that Monkeys 10 and 13 had become free from infection. Cultural studies confirmed this inference, as will appear later.

DISTRIBUTION OF BARTONELLA BACILLIFORMIS IN THE TISSUES IN THE EXPERIMENTAL INFECTION.

In Oroya fever *Bartonella bacilliformis* persists in the blood until the patient's death. Whether or not various organs and tissues also contain the living parasite has not been determined. The pronounced changes found in certain organs have been assumed to be due either to invasion by the parasite or to the action of a toxin circulating in the blood. Cultural determination of the distribution of the organism in the tissues of experimental animals was, therefore, of special usefulness in establishing a relation between the pathological processes and the presence of the parasite.· The results of cultural studies and of examination for pathological changes are recorded in parallel columns in Table I.

As the analysis shows, *Bartonella bacilliformis* was recovered from blood, liver, spleen, lymphatic glands, bone marrow, and local lesions in all instances of fatal infection, except when cultures were lost through contamination. The parasite was not so generally distributed in animals sacrificed during the course of an apparently non-fatal infection, though the local lesions and the changes in the spleen were rather severe. In Monkey 23 the parasite had disappeared from the blood, owing probably to the development of a blood immunity, but persisted in the lymphatic glands and spleen, while in Monkey 29 the blood and nodular tissue contained it but the organs were all free

TABLE I.

M. rhesus No.	Blood		Liver		Spleen		Lymph gland		Bone marrow		Nodule	
	Anemia	Culture	Degree of histological change	Culture	Degree of histological change	Culture	Degree of histological change	Culture	Degree of histological change	Culture	Present	Culture
Group 1 (fatally infected)												
17	+++	+	+++	Cont.	++++	+	++++	+	++++	+	Present	+++++
25	++++	Cont.	++++	"	++++	Cont.	++++	Cont.	+++	Cont.	"	+++++
30	++	++	++++	++	++++	++	+++	++	+	++	"	+++++
35	+	Cont.	++	Cont.	+++	Cont.	+++	Cont.	+++	Cont.	"	+++++
37	++++	Cont.	+++	Cont.	+++	Cont.	+++		+++		"	+++++
Group 2 (sacrificed during active stage of infection)												
4	...	+	-	...	++	...	+++	...	...	...	"	+++++
5	+	+	-	-	+++++	+++	+++++	++++	+++	+++	"	+++++
23	-	-	++	Cont.	<	<	<	:	:	:	"	+++++
24	++	++	++	+	?	+	++	:	:	:	"	+++++
29	++	+	-	-	+++	-	-	-	-	-	"	+++++
Group 3 (sacrificed during convalescence)												
7	+	+	-	Cont.	<+	+	++	++	-	+	Had disappeared	
8	-	-	-	-	-	-	+	+	-	-	"	
10	-	-	-	-	-	-	-	-	-	-	"	
13	-	-	-	-	-	-	-	-	-	-	"	

Anemia: ++++ = r.b.c. 1,000,000 to 1,500,000, Hb. 15 to 25 per cent; +++ = r.b.c. less than 3,000,000; ++ = r.b.c. less than 4,000,000; + = less than 5,000,000; - = over 5,500,000.

Degree of change in tissue varies from marked (++++) to slight (+).

Cont. = secondary bacterial contamination.

Summary of Pathologica

M. rhesus No.		Liver
17	Inoculation Mar. 15, 1926. Died after 57 days. Fever still present. Nodules active. Anemia marked (r.b.c. 3,112,000, Hb. 50 per cent). Blood culture + at autopsy. Nodule culture + at autopsy	Pale, somewhat enlarged. Structure d turbed, cells along central zones poor stained. Perivascular proliferation mo erate. Greenish pigment present. . *bacilliformis* found in a few endotheli cells. Culture contaminated
25	Inoculation Mar. 23, 1926. Died after 36 days. Course practically afebrile. Local lesions extensive, partly generalized. Anemia very severe (r.b.c. 1,624,000, Hb. 25 per cent). Blood (1:100,000) culture + 12 hrs. before death. Culture of nodules taken at autopsy +	Pale, soft, mottled, perhaps enlarge Structure fairly well preserved but pr nounced central necrosis throughou Numerous phagocytes, polymorphon clears, and pigments present. Hepat cells vacuolated. Culture contaminate
30	Inoculation Mar. 31, 1926. Died after 25 days. Fever present. Nodules small but active. Blood (1:10,000 dilution) culture + 3 days before death. Anemia moderate (r.b.c. 3,920,000, Hb. 60 per cent). Blood and nodules taken at autopsy yielded cultures	Pale, appears fatty. Normal structure los cells stain poorly, vacuolated and di sociated. Endothelial hyperplasia; son cells contain *B. bacilliformis*. Man phagocytes in sinusoids. Culture +
• 35	Inoculation Apr. 12, 1926. Died after 29 days. Fever still present. Nodules small but active. Anemia slight (r.b.c. 4,776,000, Hb. 80 per cent). Blood (1:100) culture + 10 days before death. Blood and nodules taken at autopsy yielded cultures	Probably paler than normal. General strut ture disturbed. Marked dissociation an vacuolation of cells. Invasion of sinu soids by phagocytes. Proliferation c endothelial cells. Culture +

and Cultural Findings.

	Spleen	Lymphatic glands	Bone marrow	Remarks
	Enlarged, rather firm. Follicles reduced. Endothelial hyperplasia. Active phagocytosis. Fibrin masses and pigments present. *B. bacilliformis* found. Culture positive	All lymph nodes much swollen and congested. Endothelial hyperplasia and active phagocytosis throughout. Culture +	Dark grayish red. Very active phagocytosis. Numerous normoblasts and polymorphonuclear leucocytes present. Culture +	*B. bacilliformis* found in all tissues studied
	Slightly enlarged, soft. Follicles smaller, outlines of many lost. Numerous perifollicular necrotic foci. Marked endothelial hyperplasia. Active phagocytosis of degenerated polymorphonuclears and erythrocytes. Many normoblasts present. Culture contaminated	Marked swelling throughout. Mesenteric nodes appear like scattered or clustered peas but bluish in color. Pronounced proliferation of endothelial cells and active phagocytosis. Culture contaminated	Dark grayish red. Active phagocytosis. Polymorphonuclears and normoblasts present. Culture contaminated	Terminal bacterial infection (pericarditis) intervened after heart puncture 24 hrs. before death
	Enlarged. Follicles thinned and diffuse in places. Endothelial elements increased around vessels; small foci with fibrinous exudate and cell débris. Culture +	General swelling. Endothelial hyperplasia, active phagocytosis, greenish pigments. *B. bacilliformis* found in a few endothelial cells. Culture +	Grayish red. Active phagocytosis. Polymorphonuclears and normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied
	Dark and soft. Follicles ill defined, pervaded by increased endothelial cells. Active phagocytosis. Culture +	Swelling and congestion throughout. Endothelial hyperplasia. Phagocytosis. Culture +	Grayish red. Active phagocytosis. Culture +	*B. bacilliformis* found in all tissues studied

Summary of Pathologica

M. rhesus No.		Liver
37	Inoculation Apr. 17, 1926. Died after 41 days. Fever still present. Small nodule on leg. Blood (1:1,000) + 96 hrs. before death; r.b.c. 1,664,000, Hb. 25 per cent. Autopsy blood contaminated; anemia extreme (r.b.c. 1,176,000, Hb. 15 per cent). Nodules (autopsy) +	Pale and soft. General structure much d turbed. Cells stain very poorly, necro along central zones. Small masses polymorphonuclear leucocytes scatter in sinusoids, where many loaded phag cytes are present. Endothelial .prolifer tion marked. Culture contaminated
4	Inoculation Dec. 8, 1925. Killed after 22 days. Fever present. Nodules active. Blood culture at autopsy +. Nodules at autopsy +	Apparently normal. No culture made .
5	Inoculation Dec. 11, 1925., Killed after 68 days. Had become afebrile. Nodules large and active. R.b.c. 4,856,000, Hb. 85 per cent. Blood culture and nodules (autopsy) +	Apparently normal. No culture made
23	Inoculation Mar. 9, 1926. Killed after 53 days. Fever present. Nodules active, extensive. Blood + in 1:100,000, 40 days previously, — 22 days previously and at autopsy. Nodules (autopsy) +	General structure irregular; some ill stain foci. Numerous polymorphonuclear Moderate endothelial hyperplasia. Cu ture contaminated
24	Inoculation Mar. 19, 1926. Killed after 24 days. Fever present. Nodules active. Anemia moderate (r.b.c. 3,432,000, Hb. 55 per cent). Blood (1:10) and nodules at autopsy +	Pale, mottled. Moderate degree of centr necrosis, uniform structure lost in place Endothelial proliferation around vessel *B. bacilliformis* found in a few endotheli cells. Culture +

and Cultural Findings.

Spleen	Lymphatic glands	Bone marrow	Remarks
Dark and firm. Only a few follicles preserved. Necrotic areas (infarctions) showing active phagocytosis found. Increase in endothelial cells. Culture contaminated	Swollen and congested. Endothelial hyperplasia. Active phagocytosis. Polymorphonuclear leucocytes present. Culture contaminated	Grayish red. Active phagocytosis and increase in polymorphonuclear leucocytes. Culture contaminated	Terminal secondary infection by strain of *B. paratyphosus* A 48 hrs. before death
Somewhat swollen and firm. Considerable increase in endothelial elements. No culture made	Enlarged and congested everywhere. Definite endothelial hyperplasia; active phagocytosis; pigments. No culture made	Not examined	Pure infection with *B. bacilliformis*
Enlarged, granular surface. Follicles ill defined in many places. A few necrotic areas. Active phagocytosis. Culture +	Moderately swollen and congested. Phagocytosis active. Hyperplasia of endothelial cells. Culture +	Grayish red. Active phagocytosis. Normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied except liver. Animal moderately resistant
Somewhat enlarged, shows numerous brown patches of infarction. Many perifollicular necrotic areas. Active phagocytosis. Pigments present. Endothelial hyperplasia. Culture +	Greatly swollen and congested. Hyperplasia of endothelial elements. Very active phagocytosis. Culture +	Grayish red. Phagocytosis active. Many polymorphonuclear leucocytes. Culture +	Note absence of *B. bacilliformis* in blood and persistence in other tissues
Enlarged, bluish red. Many follicles diffuse, interspaced by proliferated endothelial cells. Active phagocytosis. Many normoblasts. Culture +	Swollen and congested. Endothelial hyperplasia and phagocytosis. Culture +	Grayish red. Active phagocytosis. Numerous polymorphonuclears and normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied. Animal quite susceptible

M. rhesus No.		Liver
29	Inoculation Mar. 30, 1926. Killed after 28 days. Fever had been present for 2 wks. Nodules very active. Blood (1:1,000) + day before autopsy. Anemia noticeable. R.b.c. 4,704,000, Hb. 70 per cent. Blood and nodules at autopsy +	Mottled. Microscopically appears normal Culture —
7	Inoculation Dec. 21, 1925. Killed after 58 days. Fever had subsided 10 days previously. Nodule removed 30 days previously +. Blood + 8 days previously. Blood taken at autopsy +. R.b.c. 5,472,000, Hb. 80 per cent	Appears normal. Culture contaminated
8	Inoculation Dec. 30, 1925. Killed after 49 days. Fever had subsided 7 days previously. Nodules absent. Blood + 14 days previously, — at autopsy. R.b.c. 5,496,000, Hb. 80 per cent	Appears normal. Culture —
10	Inoculation Jan. 8, 1926. Killed after 40 days. Fever had subsided 14 days previously, nodules 3 wks. previously. Blood (1:100) + 30 days previously, — at autopsy. R.b.c. 6,584,000, Hb. 65 per cent	Appears normal. Culture —
13	Inoculation Jan. 19, 1926. Killed after 30 days. Fever had subsided 10 days previously. No nodules. Blood + 19 days previously, — 8 days previously, — at autopsy. R.b.c. 6,104,000, Hb. 90 per cent	Appears normal. Perivascular infiltration present. Culture —

from it. In the latter instance the organism had apparently not yet invaded the organs.

Of the four animals in which fever and local lesions had been present but had subsided, two (Monkeys 7 and 8) proved to be still carrying *Bartonella bacilliformis* in one tissue or another. Monkey 8 is especially interesting, as the latent infection was demonstrable only in the lymphatic glands. The other two animals (Monkeys 10 and 13) had

cal and Cultural Findings.

Spleen	Lymphatic glands	Bone marrow	Remarks
Enlarged, bluish red. Follicles appear to be of normal size and structure. Moderate endothelial hyperplasia. Numerous normoblasts. Culture −	Swollen and congested. Hyperplasia of endothelial elements, some phagocytosis. Culture +	Not examined	Liver and spleen had probably not yet been invaded. Animal rather resistant
Enlarged, follicles prominent. Only slight hyperplasia of endothelial cells microscopically. Follicles apparently normal. Culture +	Moderate swelling. Slight endothelial proliferation. Culture +	Grayish red. No sections. Culture +	*B. bacilliformis* found in most tissues studied: evidence of chronicity of infection
Appears normal. Culture −	Marked swelling and congestion. Hyperplasia of endothelial cells. Phagocytosis active. Culture +	Grayish yellow. Apparently normal. Culture −	*B. bacilliformis* found only in lymph nodes. Animal apparently convalescing
Probably enlarged. Microscopically normal. Culture −	Definitely enlarged, but pale. General hyperplasia. Culture −	Grayish yellow. Normal. Culture −	Had recovered from infection mildness of which was probably due to resistance of animal
Slightly enlarged (?); surface uneven, firm. Definite increase in fibrous tissues. Hyperplasia of endothelial cells. Culture −	Considerable swelling and congestion. Hyperplasia. Culture −	Grayish yellow. Apparently normal. Culture −	Recovered from mild infection. High resistance of animal noted

completely recovered, as indicated by the histological findings and proven by the negative results of cultural experiments.

Brief protocols of all the animals of the present series are recorded above.

SUMMARY.

The pathological changes observed in the organs in *Macacus rhesus* monkeys which have succumbed to severe infection with *Bartonella*

bacilliformis are similar to those found in human organs in persons dying of Oroya fever.

The characteristic changes in the liver are the zonal necrosis of the cells around the hepatic veins, involving active macrophagocytosis of invading polymorphonuclear leucocytes in the necrotic areas, and a marked endothelial hyperplasia in the sinusoids or around the portal veins. In some instances there is fatty infiltration of hepatic cells.

In the spleen persistent hyperplasia of the endothelial cells of the capillaries leads to the formation of minute foci of infarction, owing to occlusion of the lumina. The follicles are dispersed or reduced, and there is an active macrophagocytosis of cellular débris, polymorphonuclear leucocytes, and erythrocytes. In some specimens an increase in normoblasts is noted. Pigment is sometimes present.

The lymphatic system shows general progressive endothelial hyperplasia, with active invasion of macrophages which contain polymorphonuclear leucocytes, erythrocytes, and greenish or dark pigments.

In the bone marrow there is increased activity of macrophagocytes. Numerous normoblasts are found in some instances.

In one monkey, sacrificed during the course of injection, small, verruga-like nodules were found in the lungs and spleen.

Bartonella bacilliformis has been detected microscopically, though in small numbers, in all tissues showing histological changes. Parallel cultural determinations of the presence of *Bartonella bacilliformis* in the blood, liver, spleen, lymphatic glands, bone marrow, and local lesions established the relationship between the pathological conditions and the presence of the parasite. The organism seems to persist longest in the lymphatic glands. Cultural methods offer a simple and conclusive means for the determination of the presence or absence of the infecting organism.

<div align="center">EXPLANATION OF PLATES.</div>

<div align="center">PLATE 11.</div>

FIG. 1. Experimental Oroya fever. Section of liver from *M. rhesus* 25, showing a rather characteristic zonal necrosis of the cells around the hepatic veins. Giemsa's stain. × 50.

FIG. 2. The same section, × 182.

FIG. 3. Human Oroya fever. Section of liver from the case (S. A. 15) from

which the strain of *Bartonella bacilliformis* employed in these investigations was isolated. The necrotic changes of the liver cells are more diffuse than in the experimental infection, but there is a definite tendency to a central type. Giemsa's stain. × 50.

FIG. 4. The same section, × 182.

FIG. 5. Human Oroya fever. Section of liver from the collection of the Harvard School of Tropical Medicine. Courtesy of Professor R. P. Strong. This patient was free from any verruga lesions and was regarded by the Harvard Commission as a pure case of Oroya fever. The section shows the characteristic central necrosis. Note the striking resemblance between this preparation and that of the monkey liver in Fig. 1. × 50.

FIG. 6. The same section, × 182.

PLATE 12.

FIG. 7. Experimental Oroya fever. Section of spleen from *M. rhesus* 23. A splenic follicle in an infarction zone, showing a perifollicular necrosis and the invasion of the follicle by proliferating endothelial cells, endothelial phagocytes, and polymorphonuclear leucocytes. Giemsa's stain. × 182.

FIG. 8. Experimental Oroya fever. Section of spleen from *M. rhesus* 25. An area of infarction where the splenic nodule has practically disappeared. Giemsa's stain. × 182.

FIG. 9. Human Oroya fever. Section of spleen, for comparison. A splenic follicle within the infarction, showing a general disorganization, necrosis, and phagocytic invasion. Giemsa's stain. × 182.

FIG. 10. Human Oroya fever. Section of spleen from another case. The structure of spleen is greatly disturbed, shows general endothelial hyperplasia, and the presence of macrophagocytes and polymorphonuclear leucocytes. Note the striking resemblance between these lesions and those shown in Figs. 7 and 8. Giemsa's stain. × 182.

FIG. 11. Section of one of the nodules in the lung of *M. rhesus* 23. Giemsa's stain. × 50.

FIG. 12. The same section, × 182, showing the proliferation of endothelial cells within the nodule.

PLATE 13.

FIG. 13. *Bartonella bacilliformis*, colonies from a blood agar slant grown for 6 days 25°C. Smear preparation stained with Giemsa's solution for 1 hour, showing the pleomorphism of the organism. Inserted here for comparison with the intracellular forms. × 1,000.

FIG. 14. Film preparation from a nodule in the lung of *M. rhesus* 23, showing irregular masses of *Bartonella bacilliformis* within the cytoplasm of an endothelial cell. Giemsa's stain. × 1,000.

FIG. 15. Film preparation from the bone marrow (femur) of *M. rhesus* 23,

showing *Bartonella bacilliformis* within one of the endothelial cells. Giemsa's stain. × 1,000.

FIG. 16. Film preparation from the spleen of *M. rhesus* 23, showing a cell (endothelial?) containing several small masses (*Bartonella bacilliformis?*). Giemsa's stain. × 1,000.

FIG. 17. Section of an experimental skin nodule produced in *M. rhesus* with a strain of *Bartonella bacilliformis* from verruga,[11] showing a dense mass of the parasites within an endothelial cell. Giemsa's stain. × 1,000.

FIG. 18. Section of an experimental subcutaneous lesion produced in the chimpanzee[3] with the strain of *Bartonella bacilliformis* from Oroya fever, showing irregularly scattered masses of *Bartonella bacilliformis*. Giemsa's stain. ×1,000.

FIG. 19. Section of a skin lesion experimentally produced in an ourang-utan,[3] showing a small mass of *Bartonella bacilliformis* (below) and a dense mass of granules, probably of a mast cell (above). Giemsa's stain. × 1,000.

FIG. 20. Human Oroya fever. Section of lymphatic gland, showing two swollen endothelial cells, loaded with minute granules, projecting into the lumen of a capillary vessel. Giemsa's stain. × 1,000. From Case S. A. 15.

FIG. 21. Human Oroya fever. Section of lymphatic gland, showing the swollen endothelial cells lining a capillary vessel in an oblique section. These cells contain the very minute granules first described by the Harvard Commission as an intracellular phase in the life cycle of *Bartonella bacilliformis*. (Courtesy of Professor Strong.) Giemsa's stain. × 1,000.

PLATE 14.

FIG. 22. The lungs of *M. rhesus* 23, showing the pale grayish nodules on both lungs. They measured 2 to 3 mm. in diameter. In the middle, near the trachea, is a grayish lymph node of the size of a pea. Natural size.

FIG. 23. The spleen of the same monkey, showing numerous infarctions. Natural size.

FIG. 24. *Bartonella bacilliformis* in an endothelial cell in a film preparation from one of the nodules in the lung of *M. rhesus* 23. Giemsa's stain. × 1,750.

FIG. 25. *Bartonella bacilliformis* in an endothelial cell. Film preparation from the spleen of *M. rhesus* 23. Giemsa's stain. × 1,750.

FIG. 26. *Bartonella bacilliformis* (?) in an endothelial cell of the bone marrow (femur) of *M. rhesus* 24. Film preparation. Giemsa's stain. × 1,750.

FIG. 27. *Bartonella bacilliformis* in an endothelial cell of the lymph gland of *M. rhesus* 29. Section. Giemsa's stain. × 1,750.

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[11] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

Liver, *M. rhesus* 25. × 50.
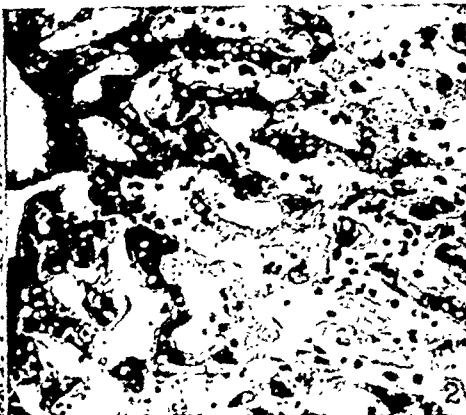
Liver, *M. rhesus* 25. × 182.
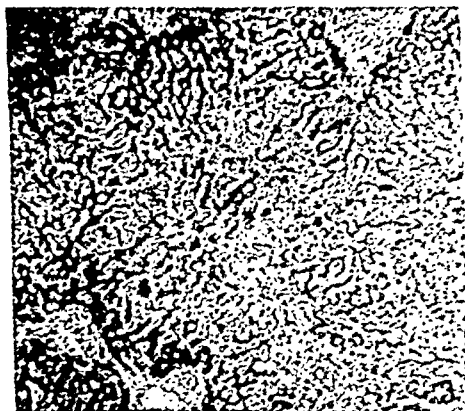
Liver, human Oroya fever. × 50.
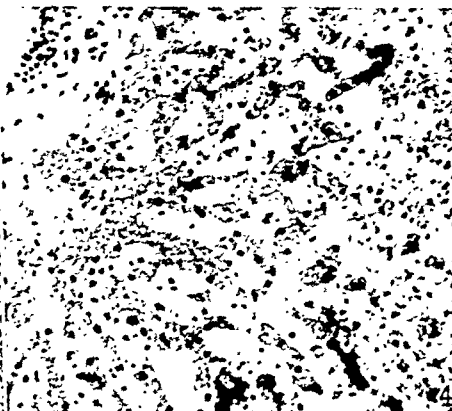
Liver, human Oroya fever. × 182.

Liver, human Oroya fever. × 50.
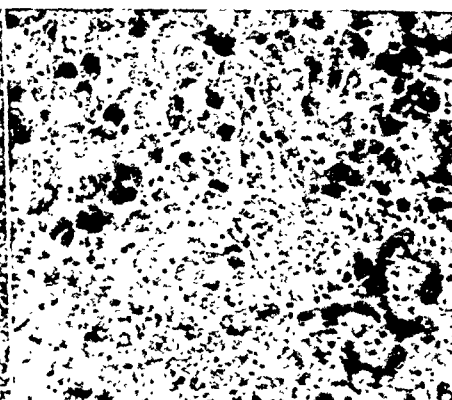
Liver, human Oroya fever. × 182.
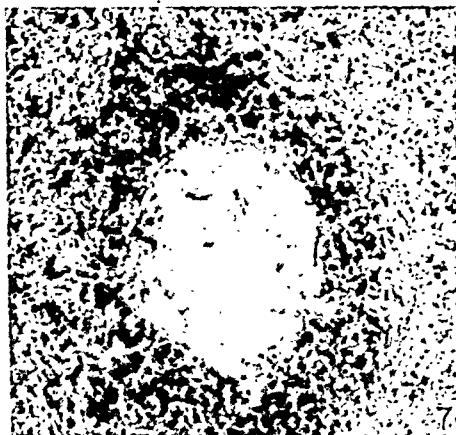
Spleen, *M. rhesus* 23.   × 182.
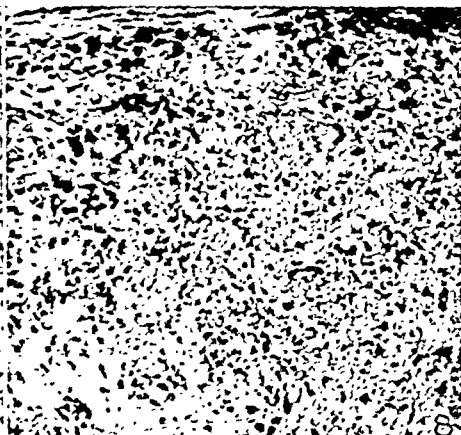
Spleen, *M. rhesus* 25.   × 182.
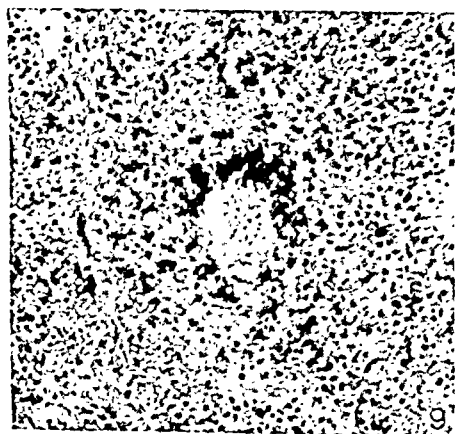
Spleen, human Oroya fever.

Spleen, human Oroya fever.

Lung nodule, *M. rhesus* 23.   × 50.

Lung nodule, *M. rhesus* 23.   × 182.

(Noguchi: Etiology of Oroya fever. VI.)
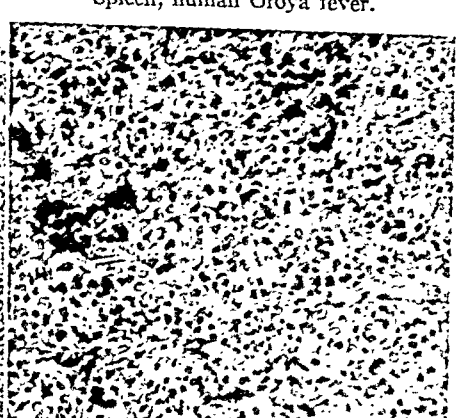
Bartonella bacilliformis. Giemsa's stain.   × 1,000.

In culture, for comparison.
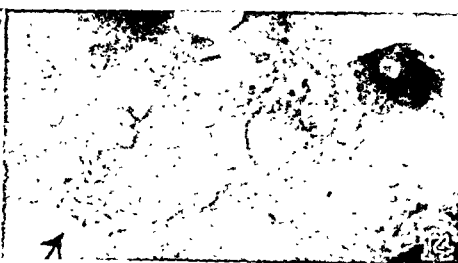
In lung nodule, *M. rhesus* 23.
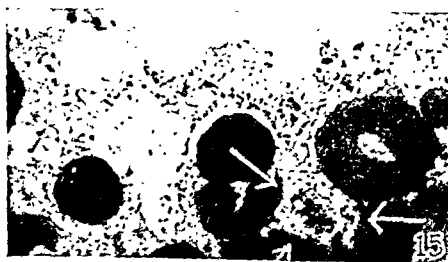
In bone marrow, *M. rhesus* 23.

In spleen, *M. rhesus* 23.

In skin nodule, *M. rhesus*.

In skin nodule, chimpanzee.

In skin lesion, ourang-utan.
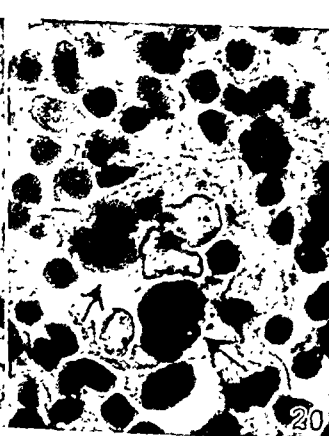
In human lymphatic gland.

In human lymphatic gland.

Lesions in lungs and spleen, *M. rhesus* 23.

Bartonella, lung nodule, No. 23.

Bartonella, spleen, No. 23.

Bartonella (?), bone marrow, No. 24.
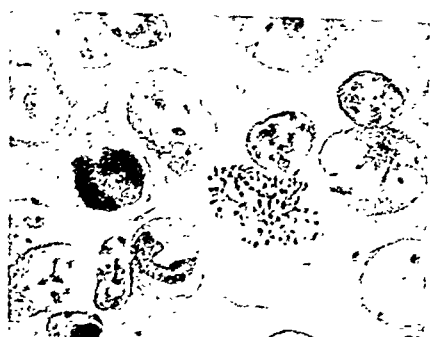
Bartonella, lymph gland, No. 29.

(Noguchi: Etiology of Oroya fever. VI.)

ETIOLOGY OF OROYA FEVER.

VII. The Response of the Skin of Macacus Rhesus and Anthropoid Apes to Inoculation with Bartonella Bacilliformis.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In animals experimentally infected with *Bartonella bacilliformis*,[1-4] spontaneous involvement of the skin is far less frequent, and rarely so extensive, as in human verruga. In man the organism probably enters the body through the skin, and, after a preliminary multiplication elsewhere—perhaps in the lymphatic glands—invades the skin from within, giving rise to a persistent endothelial hyperplasia. In animals, when *Bartonella bacilliformis* is introduced locally, a lesion arises which may in time assume dimensions as great as the human verruga nodule. But notwithstanding the persistence of the lesion for many months, and the fact that the surrounding skin is frequently bathed in blood resulting from mechanical injury to the lesion, no new nodules develop. Hence it becomes of interest to determine whether the resistance of the skin in monkeys is due to an anergic condition, such as exists in syphilis, or to inability of the parasite to invade the intact skin.

Two other questions presented themselves in this connection, (1) whether it would be possible to induce verruga formation by injuring an area of normal skin in an animal having active skin nodules elsewhere and carrying *Bartonella bacilliformis* in the blood, and (2) whether autoinoculation would be successful in actively infected

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

[4] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

animals, *i.e.*, whether skin immunity develops during the course of infection, and if so, at what time.

Resistance of the Intact Skin to Bartonella bacilliformis.

The question whether *Bartonella bacilliformis* is capable of inducing lesions when applied to the surface of the intact skin is one of practical importance.   To test the point, we applied to intact areas of skin whatever material was being inoculated elsewhere.   The infectious material was rubbed on two separate areas, each several cm. square, and usually near the sites of inoculation by scarification or intradermal injection. No cutaneous lesions were ever induced by this method, though lesions invariably developed at the sites of the intradermal injections and in many instances also on the areas inoculated by scarification.

Although unusually extensive lesions (*verruga mular*) are often produced by application of infectious material to scarified areas of the skin, there have been many instances in which this mode of inoculation failed, while typical lesions (*verruga nodular*) always arose at the sites of intradermal injection.   Hence it is desirable always to use both methods of inoculation.

Chimpanzee,[3] *Pan leucoprymnus*, about 4 years old.   *Jan. 19, 1926*, abdominal skin on the right side was shaved and two areas, each about 3 × 4 cm. square, were scarified and smeared with a saline suspension of the skin nodule* of *M. rhesus* 3.   At the same time two adjacent unscarified areas were rubbed with the same suspension.   *Mar. 1, 1926*, pinkish, raised indurated lesions had become noticeable on both scarified areas.   *Bartonella bacilliformis* was demonstrated in the lesions, both in sections and by culture.   No lesions developed on the areas which were not scarified before being smeared with the suspension.

Ourang-utan,[3] *Pongo pygæmus*, about 3 years old.   *Apr. 17, 1926*, abdominal skin shaved and an area (2 × 3 cm.) on the right lower quadrant scarified and rubbed with a piece of nodule from the eyebrow of *M. rhesus* 23.   An unscarified area on the upper quadrant was vigorously rubbed with the same material. *May 20, 1926*, pinkish, raised, linear lesions, rather pale, have been present on the scarified area for some time.   *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections.   No lesions developed on the unscarified smeared area.

M. rhesus 18.[2]   *Feb. 15, 1926*, right eyebrow and skin of right abdomen shaved and scarified and a saline suspension of the nodule of *M. rhesus* 5 applied.   Adja-

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* Nodules were removed under ether anesthesia.

cent unscarified areas were rubbed with the same material. Extensive nodules of deep red color developed on the scarified area of the eyebrow within a few weeks and numerous punctiform red miliary nodules on the scarified abdominal skin. The abdominal lesions disappeared within a month, the nodules on the eyebrow remained until the middle of June, when they became small, pale, and fibrous. *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections. No lesions developed on the unscarified smeared areas.

M. rhesus 23.[5] *Mar. 9, 1926,* left eyebrow shaved and a scarified area smeared with a piece of the nodule from the eyebrow of *M. rhesus* 18. An adjacent unscarified area was vigorously rubbed with the same piece of tissue. Within 2 to 3 weeks large protruding lesions had appeared on the scarified area, and these progressed to considerable size during the following weeks. *Bartonella bacilliformis* was found in the sections of the lesion. No lesion appeared outside of the scarified area.

Relation of Injuries to Localization of Lesions.

Whether or not mechanical factors—friction, exposure to minor injury—are involved in the spread of the skin lesions in human verruga is not known. In animals these devices appear to have no influence, as shown by repeated failure to induce localized lesions in infected animals by scarifying the skin or introducing foreign substances (agar, culture medium) intradermally during the height of the infection, when *Bartonella bacilliformis* was demonstrable in the blood. In one unusually susceptible animal, *M. rhesus* 25,[2] in which spontaneous miliary nodules developed, scarification of the abdominal skin had no influence on the course of the general eruption. Similar attempts to induce nodule formation failed also in the chimpanzee, the ourang-utan, and in *M. rhesus* 18,[2] which had at the sites of scarification on the eyebrow one of the most extensive local lesions (*verruga mular*) observed in the course of the experimental work with *Bartonella bacilliformis.*

It is evident that in experimental animals *Bartonella bacilliformis* cannot be made to localize in an area of skin injured either by scarification or by intradermal inoculation of foreign substances, though present in the circulating blood and in the lymph channels, as well as in the skin lesions. Injuries of the skin are not equivalent to the deposition of concentrated infectious material on the scarified skin or in the cutaneous tissues.

[5] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

Autoinoculability and Superinfection.

In a disease like verruga peruana, which persists over a long period, there must be a steady process of self-infection.   The manner in which the cutaneous lesions spread has not been definitely determined, but it is certain that the skin must retain its susceptibility during periods of remission and relapse.   The skin of *Macacus rhesus* and that of the apes is relatively so insusceptible to infection with *Bartonella bacilliformis* that spontaneous eruptions (*verruga miliar*) seldom occur, but it has been possible to induce well marked nodular lesions (*verruga nodular*) in actively infected animals by intradermal inoculation of suspensions of nodular tissue either from the same or from another animal.   Such nodules never attained the size of the primary lesions, and they usually began to recede earlier.   In no instance was it possible to induce any lesions on scarified areas during the course of infection, though the materials employed induced typical lesions in control animals.

In infections with the strain of *Bartonella bacilliformis* derived from Oroya fever, the existence of active lesions considerably reduced the susceptibility of the skin to subsequent inoculation.   Moreover, once the animals were free from *Bartonella bacilliformis,* they were completely refractory to reinoculation, hence the reduction in susceptibility during the course of illness is due to a partial immunity, not to a state of anergy.

On the other hand, in infections with the strain of *Bartonella bacilliformis* from a case of verruga,[6] there have been exceptional instances in which a preexisting infection did not prevent the development of the most severe type of local infection after reinoculation with the same (verruga) strain.   *M. rhesus* 33 and *M. rhesus* 34 showed this type of reaction.

M. rhesus 33 was inoculated on Apr. 7, 1926, with the saline suspension of a human verruga nodule (Case P 5).   Blood taken on Apr. 16 and again on May 27 yielded (in 1:10 dilution) cultures of *Bartonella bacilliformis.*

On June 3 the animal was inoculated intradermally and by scarification with the suspension of a nodule from the eyebrow of *M. rhesus* 41 (second generation of the same strain).   On June 24 the lesions had already developed at the sites of inoculation, and by July 1 they were very large and active.

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[6] Noguchi, H., *J. Exp. Med.,* 1927, xlv, 175.

M. rhesus 34 was inoculated on Apr. 10, 1926, with the saline suspension of the nodule of Case P 5. Cultures was obtained from diluted (1:10) blood on April 28, May 12, and May 27. By June 29 a large nodule had developed on the tail.

On June 3 the animal was inoculated intradermally and by scarification with the suspension of the eyebrow nodule of *M. rhesus* 41. *Bartonella bacilliformis* was cultivated from a 1:100 dilution of blood taken on June 20. Large lesions had appeared at the sites of inoculation at this time, and by July 1 the lesions had become unusually extensive.

As these instances show, it is not possible to make any general statement with regard to the partial immunity observed in most animals when passing through a protracted course of infection with *Bartonella bacilliformis*. Immunity may develop so slowly in some animals as not to interfere with the course of a superinfection.

Pathogenesis of the Skin Lesions.

The verruga-like skin lesion produced by *Bartonella bacilliformis* in anthropoid apes and *M. rhesus* is a slowly progressing infectious angioendothelioma and owes its origin to a delicate reciprocal relation between the endothelial cell and the microorganism. *Bartonella bacilliformis* finds within the cytoplasm of endothelial cells most favorable conditions for its multiplicative phase, and once lodged there multiplies steadily, though perhaps rather slowly, eliciting certain reactions on the part of the infected cells. Since these parasitized cells remain alive for a long time and undergo active mitosis, it is clear that the parasite furnishes a mild type of stimulus which leads to continuous proliferation of the cells. The lesion thus started continues to grow until local as well as general immunity intervenes to terminate the peculiar association of cells and parasite.

The evolution of the verruga lesion in experimental animals may be divided into (1) the incubation period, (2) the initial stage, (3) the mature vascular stage, and (4) the regression. These divisions are of course arbitrary, one stage passing into the next imperceptibly, yet each at its height has its special feature.

Incubation Period.—No instance has been observed in which the lesion was macroscopically recognizable within less than 10 days after inoculation. Usually a trace of induration can be detected after 10 to 14 days, though in some cases the incubation period is as long as 3 weeks.

Initial Stage.—When the nodular lesion, which becomes noticeable as a slightly raised area, 1 to 2 mm. in diameter, has reached a size of 4 to 6 mm. and protrudes 2 to 3 mm. beyond the surrounding normal skin, the color becomes slightly pinkish, and edema of the surrounding tissues becomes evident. The nodule is now firm and well defined to the touch and can be easily separated from the overlying skin and the loose connective tissue in which it is embedded. The cut section is grayish pink, edematous, but not soft, and microscopically the nodule is seen to consist of masses of endothelial cells, placed close together, lying among capillary vessels and connective tissue fibers. A small number of lymphocytes and polymorphonuclear leucocytes are seen in the interstices of the cutis, but not within the nodule itself. The endothelial walls of the capillaries are thickened, and the lumina of the blood vessels narrowed. Practically every endothelial cell in the nodule contains *Bartonella bacilliformis*, some cells being packed with clumps of organisms. The endothelial cells lining the capillaries are often filled with similar masses of microorganisms. The histological picture at this stage is very simple.

Mature and Vascular Stage.—The interval from the preceding stage to this one varies from a few to 10 to 14 days and persists for 10 days to 2 weeks.

The increase in size of the nodule may be gradual or even relatively rapid until the lesion measures 8 to 10 mm. or more in diameter. The color first becomes bright, then deep, red. The overlying skin is shiny, and the epidermis scaly in places. The nodule is still firm and is sharply demarcated from the surrounding tissues. The color may change to a dark bluish red, suggesting that of a cherry. This type of lesion, especially when deep seated and less vascular on the surface, is characteristic of the form of human lesion known as *verruga nodular*. Occasionally the overlying skin yields to the increasing pressure from within, and the lesion becomes pedunculated.

The lesions found on the scarified lines of the skin are seldom round; on the contrary, they are usually irregular at the surface, and the mass resembles a raspberry in form and color, being made up of a number of small, translucent, red, drupelet-like nodules. The entire region adjacent to the lesion is edematous. The lesion is devoid of an epidermic layer, hence it bleeds readily on slight friction. This type of lesion corresponds to that termed in man *verruga mular*.

In a few animals spontaneous nodules have appeared on the skin at sites remote from those inoculated; these correspond to the general eruption in human cases known as *verruga miliar*. Such lesions have always been small, none becoming larger than 1 to 2 mm. in diameter, but their clinical course is similar to that of the nodules induced by intradermal inoculation or by scarification.

On microscopic examination, nodules or lesions removed during the mature and vascular stage present a somewhat more complex picture than those of the initial stage. The large number of capillary vessels intersecting dense masses of closely packed endothelial cells and the increase of fibrous connective tissue irregularly penetrating the nodular structure are striking features of the lesions. Numerous fibroblasts and fibrils lie between islands of angioblasts, the appearance somewhat suggesting that of a fibrosarcoma. In places there is hyperplasia of the epidermis, and some large detached masses of epithelioid cells find their way deep into the nodular zone, a circumstance which complicates the interpretation of the cellular elements composing the nodule. Some of these invading epithelioid cells show hydropic degeneration. Degenerating endothelial cells and numerous erythrocytes are present in some places and are taken up by migrating macrophages, which also engulf the dead polymorphonuclear leucocytes simultaneously present in these areas. Along the interstitial spaces penetrated by connective tissue fibers, plasma cells and mast cells are sometimes rather numerous. The lumina of the capillary vessels are often occluded or compressed by proliferating endothelial cells. Mitotic figures are common. The skin may be adherent.

The microorganisms are more uniformly present in the endothelial cells along the periphery of the nodule than among the cells in the older foci. None except endothelial cells contain *Bartonella bacilliformis* in the cytoplasm, and no extracellular localization of the parasites can be detected.

When a secondary bacterial invasion has taken place, there are found, needless to say, an enormous number of polymorphonuclear leucocytes loaded with the contaminating bacilli or cocci. At times these bacteria-carrying leucocytes are taken up by endothelial cells and give rise to a confusing appearance, as though the secondary invaders were the primary cause of the hyperplasia.

Regression.—The time of beginning of the regressive process depends on the individual animal's resistance to the infection, and the process of regression is intimately associated with the acquired immunity, local or constitutional.   Early regression, before full development of the lesion, has been rather frequent in the experimental animals.   Once the nodule has fully developed, however, considerable time is required for its complete resolution, 4 to 5 months in some instances.   The first indication of retrogression is the gradual loss of the deep red color.   As the color fades the size of the nodule begins to diminish, the paling and diminution continuing until finally only a tiny colorless wart remains. When the nodule ruptures spontaneously at the beginning of the period of regression, the whole mass sloughs and the wound heals rapidly with scar formation.   The result is quite different, however, when the nodule is injured, or partially removed by surgical operation during its developing stages; in this instance the lesion acquires renewed vigor and spreads far beyond its previous limits in the form of a *verruga mular;* it becomes malignant.

The histological appearances of the lesions, as just presented, are similar to those observed by the Harvard Commission[7] in human and experimental verruga tissues, of which they carefully studied a large number.   The eosin-staining inclusions so constantly encountered in the endothelial cells, both in human and monkey nodules, and particularly emphasized by the Commission, are of considerable significance.   These reddish granules, which may have been identical with those described by Mayer, Rocha-Lima, and Werner[8] as Chlamydozoa, in all probability represent intracellular forms of *Bartonella bacilliformis,* somewhat less distinctly stained than in our preparations. The possibility of indistinct staining is one which cannot be ruled out in connection with this difficultly staining microorganism.

[7] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[8] Mayer, M., Rocha-Lima, H., and Werner, H., *Münch. med. Woch.,* 1913, lx, 739.

SUMMARY.

Bartonella bacilliformis failed to induce lesions when merely rubbed on the surface of the intact skin of a chimpanzee, an ourang-utan, and numerous *Macacus rhesus* monkeys, although when applied to the scarified skin of the same animals it gave rise to extensive lesions.

Application of infectious material to the scarified skin did not always induce verruga lesions, but intradermal inoculation almost invariably gave rise to nodule formation.

The localization of *Bartonella bacilliformis* in the skin is not, in experimental animals, determined by mechanical factors, since scarification of the skin or intradermal injection of foreign substances in monkeys infected with *Bartonella bacilliformis* does not give rise to verruga formation.

The degree of susceptibility of the skin tissues appears to be considerably diminished during the course of experimental infection with *Bartonella bacilliformis*. Inoculation of the scarified skin of infected animals gave uniformly negative results, and intradermal inoculation induced only a mild local reaction. In a few exceptional instances, however, of animals previously infected with the strain of *Bartonella bacilliformis* derived from a human verruga nodule, reinoculation with the same strain gave rise to unusually marked reactions.

The evolution of the skin lesion induced in experimental animals by *Bartonella bacilliformis* may be divided into four stages, the period of incubation, the initial stage, the mature and vascular stage, and the regression. In the initial stage the lesion is a pure angioendothelioma, but in the stage of full development the histological picture is complicated by connective tissue proliferation and occasionally also by penetration of epidermis into the lesion. The demonstration of *Bartonella bacilliformis* in the endothelial cells distinguishes the lesion from others which simulate it.

The cutaneous lesions known as *verruga nodular*, *verruga mular*, and *verruga miliar* have been reproduced in monkeys.

EXPERIMENTS ON THE PRODUCTION OF WASSERMANN REAGINS BY MEANS OF TRYPANOSOMES.

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According to the original view of the discoverers the Wassermann reaction was considered, in analogy to the common serological reactions, as being caused by antibodies specific for the infecting microorganisms. This view became doubtful and was abandoned by most authors when it was shown that syphilitic sera react with alcoholic extracts of organs, presumably with lipoids present in these extracts (1–3). The use of extracts of normal instead of syphilitic organs (1) has since been accepted almost generally for the technique of the Wassermann reaction. Several main hypotheses have been advanced in order to explain the formation and properties of the Wassermann reagins. Some authors believed that the action of the syphilitic sera was due not to immune bodies but to non-specific changes of the serum proteins. Others regarded the reagins as antibodies formed either by autoimmunization with lipoidal substances from the infected organism or with lipoids derived from the spirochetes (*cf.* Bergel). In the latter case one had to assume that the antibodies produced act not only upon the homologous antigen but also upon lipoids of very different origin.

The hypotheses implying an immunization against lipoids met with the difficulty that the attempts to induce the formation of antibodies by injection of lipoids, as a rule, did not yield clear-cut and consistent results. In the positive experiments recorded there was no certainty as to the absence of small quantities of antigenic proteins in the material injected or else the amount of antibody formation was rather insignificant.[1]

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[1] Regarding the antigens of blood corpuscles see "On the antigens of red blood corpuscles. The question of lipoid antigens," by K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, 1925, xli, 427.

A new impulse was given to the study of lipoid antigens by the work on the Forssman's heterogenetic reaction. It led to the view that Forssman's antigen has a complex constitution and is composed of a specific part binding but not immunizing, which part can be extracted by alcohol, and of a protein responsible for the immunizing effect (4-6). Moreover by means of a special method regular results in the immunization with alcoholic extracts of Forssman's antigen (horse kidney) could be obtained (5, 7). These extracts became antigenic when mixed with protein solutions such as human or pig serum, although in themselves they lack this property or exhibit it to a very small degree only. The case of the Forssman antigen is not exceptional as was shown by the application of the same method to the alcoholic extracts of erythrocytes (8). Evidently the findings reported suggested the view that the antigen operative in the production of Wassermann reagins may possibly be of a complex structure analogous to that of the heterogenetic antigen (5, p. 305).

The statement made by Taniguchi (9) that animals treated with Forssman's antigen may develop Wassermann reagins beside the hemolytic antibodies offered no satisfactory explanation since organs free from Forssman antigen did not produce a like effect in his experiments.

Sachs, A. Klopstock and Weil (10) emphasized a hypothesis related to that of Citron. They assume that the change in the serum is due to the action of a combination of lipoids of the tissues with proteins of the spirochetes. The authors actually were able to show that, in analogy to the experiments on heterogenetic antigen, after injections of alcoholic extracts of rabbit organs along with pig serum into rabbits the sera of the animals became Wassermann-positive.

The idea that the microbes contain the antigen responsible for the characteristic alteration of the serum was examined by F. Klopstock (11) and by ourselves (12). In Klopstock's experiments the Wassermann-positive sera were got by injections with killed *Spirochæta pallida*, in ours with killed *Trypanosoma equiperdum*. The use of trypanosomes for the study of the Wassermann reactions is warranted by the fact that infections with trypanosomes often result in the appearance of a positive Wassermann reaction (13, 14) which is

apparently equivalent to that of syphilitic sera. The occurrence of these reactions in rabbits seems to vary considerably with different strains of trypanosomes according to our experience. (See Browning and McKenzie (15).)

The results of our investigation briefly described in a preliminary communication (12) are given more fully in the present paper.

EXPERIMENTAL.

Rabbits were injected with a suspension of dead trypanosomes (*Trypanosoma equiperdum*) prepared as follows: Citrated blood taken from rats at the height of the infection was diluted with an equal volume of saline and the erythrocytes sedimented by gentle spinning. The supernatant fluid and the resuspended white layer on top of the blood corpuscles were joined and the trypanosomes sedimented by vigorous centrifugalization. The sediment was washed once with about 25 times its volume of saline. A suspension was made with about 4 cc. of saline for every rat used. This turbid fluid was rich in live trypanosomes and contained few blood cells when examined microscopically. After addition of one-twentieth of its volume of 5 per cent phenol it was kept in the ice box overnight. By that time the microbes were largely disintegrated. No infections with trypanosomes occurred upon administration of even large doses of this material.

The strain of trypanosomes employed was of low virulence for rabbits, that is, a very chronic disease developed. The lesions, however, were intense and typical.

In all the experiments special care had to be taken in selecting the animals owing to the frequent occurrence of positive reacting sera in stock rabbits. Therefore the sera were tested with the flocculation method and the complement fixation with cholesterolized beef heart extract and with emulsions of commercial egg lecithin (Merck). The animals were used only if these tests were completely negative, or, in some cases, gave very weak reactions with the Sachs-Georgi method with serum diluted 1:2. In order to pick out enough rabbits for the experiments the selection had to be made from a rather great number of animals.

Wassermann Tests.—To 0.25 cc. of progressively doubled dilutions of inactivated serum starting with 1:5 was added 0.25 cc. of an emulsion of cholesterolized beef heart extract and 0.25 cc. of 1:10 guinea pig serum. After incubation for 1 hour at 37°C. 0.25 cc. of diluted sheep blood immune serum containing 2¼ to 3 hemolytic units and 1 drop of 50 per cent sheep blood were added.

The antigen was prepared by extracting beef heart with 5 volumes of alcohol 95 per cent and adding 0.06 of the volume of a 1 per cent alcoholic solution of cholesterol. From this an emulsion was made by adding first 1 volume and after about 10 seconds 4 volumes of saline solution.

Sachs-Georgi Tests.—0.2 cc. of the inactivated serum diluted 1:2 and 1:10 were mixed with 0.2 cc. of the emulsion of cholesterolized beef heart extract. The tubes were kept at 37°C. for 2 hours and at room temperature overnight.

TABLE I.

Injections with Dead Trypanosomes.   The Animals Were Tested on the Same Day, after They Had Been Injected 4 to 7 Times.

No. of animals	Before injection: Sachs-Georgi flocculation with serum diluted 1:2	After injections with dead trypanosomes							No. of injections
		Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes		
			2	10					
1	0	0,0,tr,vstr	+++	+±	ac,c,c	0	0,0,str,c		5
2	0	0,0,0,d,ac,c	+++	+±	c,c,c	0	0,0,0,str,ac		5
3	0	0,0,0,0,str,c	++++	+++	str,vstr,ac,c	0	0,0,0,0,str		4
4	tr	w,d,str,c*	+*	0	vstr,d,str,ac,c	ftr	0,0,w,ac,c		7
5	0	0,0,0,0,d,c	+++	+++	ac,ac,c	0	0,0,0,tr,c		6
6	0	0,0,0,0,0,str	+++	+++	w,0,w,ac,ac,c	+	0,0,0,0,vstr		4
7	tr	0,0,vstr,c	++	+±	d,str,c	±	0,0,0,str,c		5
8	0	d,w,str,ac,c	++	+	c,c,c	0	0,0,ftr,tr,ac,c		7

* Tests after 7 injections.   The reactions with this serum were strongly positive after 4 injections.

Lecithin Tests.—For the complement fixation 1 part of a ½ per cent alcoholic solution of egg lecithin Merck was emulsified by fairly rapid addition of 24 parts of saline solution. The fluid is opalescent. The complement fixation tests were made as described above. This way of preparing the emulsion was found necessary to avoid reactions with normal sera.

For the flocculation tests (with inactivated serum 1:2) the emulsion was prepared by rapid addition of 5 parts of saline solution to 1 part of a ½ per cent

TABLE II.

Sera of Rabbits Infected with Trypanosomes Tested 1 Month after the Infection

No. of animals	Before infection	1 mo. after the infection with trypanosomes					
	Sachs-Georgi flocculation with serum diluted 1:2	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes
			2	10			
9	0	0,0,0,0,str,c	+++	+±	0,0,0,str,c	++±	tr,d,str,c
10	0	ac,ac,ac,c	+±	0	c,c,c	±	vstr,vstr,ac,ac
11	0	str,str,ac,c	+	ftr	0,0,c	+±	vstr,vstr,ac,ac
12	0	0,0,0,ac,c	+++	+	0,0,ftr,ac,c	++±	tr,tr,str,vstr,c
13	0	str,str,vstr,c	+±	±	w,0,w,ac,ac,c	++	ac,ac,c
14	±	0,0,0,tr,c	+++±	+	0,0,0,0,ac,c	++	str,str,vstr,ac,c
15	0	0,0,vstr,c	+	tr	0,0,vstr,c	+±	vstr,ac,ac,c

Normal Rabbits.

A		c,c,c,c	0		c,c,c,c	0	c,c,c,c
B		c,c,c,c	0		ac,c,c,c	0	c,c,c,c

The tests recorded in Tables I and II were made on the same day and with the same emulsions. For comparison the reactions of sera of normal rabbits are recorded.

solution of the lecithin. The readings were taken after 20 hours at room temperature.

Tests with Trypanosome Extract.—1 volume of the packed microbes was extracted 24 hours with 25 parts of 95 per cent alcohol. The filtered solution was emulsified by fairly rapid addition of 5 parts of saline solution and the emulsion was used for complement fixation as in the Wassermann tests.

The strength of the reactions in these tests is indicated as follows: Hemolysin tests—0 = no hemolysis, ftr = faint trace, tr = trace, w = weak, d = distinct,

TABLE III.—*8 Rabbits Were Injected with Dead Trypanosomes on April 24, 30, and May 5,*
May 5

No. of animals	Before injection					After 2 injections with dead try			
	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:2	Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin
							2	10	
16	c,c,c,c	0	c,c,c,c	0	ac,c,c,c	vstr,ac,c	tr	0	ac,ac,c
17	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,w,c	+++±	±	c,c,c
18	c,c,c,c	0	c,c,c,c	0	c,c,c,c	d,str,c,c	+	0	ac,c,c
19	c,c,c,c	tr	c,c,c,c	0	c,c,c,c	vstr,ac,c	±	0	c,c,c
20	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,d,c	+	tr	vstr,ac,c
21	c,c,c,c	ftr	c,c,c,c	0	ac,c,c,c	0,0,0,d,c	+++	++	ac,ac,c
22	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,w,ac,c	+++	+	ac,c,c
23	c,c,c,c	0	c,c,c,c	0	c,c,c,c	d,w,vstr,ac	ftr	0	c,c,c,c

Normal Rab

C						c,c,c	0	0	ac,c,c
D									

* Tests 13 days after the 1st injection.

with Doses Corresponding to 6 or 7 Mg. Dry Weight of Trypanosomes. The Tests Were Made on and 13.

panosomes (after 11 days)		After 3 injections with dead trypanosomes (after 19 days)						
Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	
			2	10				
0	0,w,ac,c	0,0,0,0,0,ftr,ac,c	++	+++±	c,c,c,c	0	0,0,0,0,w,vstr,ac	
tr	0,0,w,vstr	0,0,0,0,0,0,vstr,ac	+++	+++	c,c,ac,c	0	0,0,0,0,0,w,ac	
0	0,tr,vstr,c	0,0,0,0,str,c,c	+++	++±	c,c,c,c	+±	0,0,tr,vstr,c	
0	str,vstr,d	0,0,0,0,vstr,c	+++	+±	c,c,c,c	±	0,0,str,ac,c	
0	0,0,d,ac,c	0,0,0,0,vstr,c	+±	±	c,c,c,c	0	0,0,d,ac,c	
0	0,0,0,str,c	0,0,0,ftr,ac,c	+++	++±	c,c,c,c	0	0,0,0,0,w,c	
0	0,0,tr,c	0,0,0,0,0,0,d,ac	+++	+++	c,c,c,c	tr	0,0,0,0,ftr,vstr,c	
0	tr,str,c*	str,d,vstr,ac,c	ftr	0	c,c,c,c	0	0,0,0,vstr	

bit Sera.

0	c,c,c	c,c,c	0	0	c,c,c	0	c,c,c	
		ac,c,c	0	0	c,c,c	0	c,c,c	

TABLE IV.

Tests with Sera of 7 Rabbits Infected with Trypanosomes.

The table reproduces the results at the time when the strongest reactions were observed. In the last column the number of days after the infection is given.

No. of ani-mals	Before infection					After infection						
	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi floccu-lation with serum diluted 1:2	Complement fixation with lecithin	Floccu-lation with lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation 1:2	1:10	Complement fixation with lecithin	Floccu-lation with lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Days after infec-tion
24	c,c,c	tr	c,c,c	0	ac,c,c,c	0,0,0,0,str,c	+++	+++	0,0,0,ftr,str,ac,c	+++	tr,ftr,0,w,str	32
25	ac,ac,c,c	tr	c,c,c,c	0	c,c,c	0,0,0,ftr,vstr	+±	+	0,0,0,tr,ac,c	+	w,w,d,str,ac	32
26	c,c,c	0	c,c,c	0	c,c,c	vstr,ac,c,c	tr	ftr	ac,c,c,c	0	ac,c,c,c	22
27	ac,c,c	0	c,c,c	0	c,c,c	0,0,0,0,0,vstr,c	+++	+++	0,0,0,0,0,c	+++	0,tr,w,vstr,ac,c	50
28	ac,c,c	0	c,c,c	0	c,c,c	d,tr,str,ac	tr	0	w,str,ac,c	+	d,str,vstr,c	22
29	vstr,c,c	0	c,c,c	0	c,c,c	0,0,0,vstr,ac,c	+++	++	0,0,0,str,c	+++±	vstr,str,ac,c,c	42
30	c,c,c	0	c,c,c	0	c,c,c	0,0,vstr,ac,c	+±	±	0,0,w,ac,c	++	d,w,vstr,ac,c	42

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str ≈ strong, vstr = very strong, ac = almost complete, c ≈ complete hemolysis. *Flocculation tests*—0 = no flocculation, ftr = faint trace, tr = trace; ±, +, ++, +++, etc.

In all experiments positive sera of known strength and negative ones were used as a control.

In our first experiments 7 rabbits were infected by injection of 1 cc., 50 times diluted blood from an infected rat, and 8 rabbits re-

TABLE V.

Human Syphilis Sera.

Nos. of the sera	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi tests with serum diluted 1:		Complement fixation with lecithin	Floccu-lation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes
		2	10			
S₁	0,0,0,0,w,vstr,c	+++	+++	ac,c,c,c	±	str,d,str,vstr,ac
S₂	0,0,0,0,tr,d,str	+++	+++	c,c,c,c	+	ac,vstr,ac,c
S₃	0,w,vstr,c	±	0	vstr,vstr,ac,c	±	
S₄	0,tr,ac,c	+++	++	c,c,c,c	+	c,c,c,c
S₅	0,0,0,d,c	++	+	c,c,c,c	+±	vstr,ac,c
S₆	0,0,0,d,c	+++	++	c,c,c,c	+	ac,ac,c

Rabbit Syphilis Sera.

50	0,0,0,w,ac,ac	+++	++±	0,0,d,ac,c	++±	tr,str,c,c
51	0,0,w,ac	+++	±	tr,str,c	±	
52	0,0,w,c	++±	++±	0,tr,str,ac	++	
53	0,ac,c,c	++±	ftr	d,str,c	0	
54	0,0,0,tr,vstr,ac	++++		0,tr,str,ac,c	++±	0,0,tr,vstr,ac
55	0,0,tr,vstr,vstr	+++	+	0,tr,vstr,ac,c	+	ftr,ftr,d,vstr,c

ceived every 4 to 7 days intravenous injections of 3½ cc. of an emulsion of killed trypanosomes, containing 6 to 7 mg. of dry material.

Table I shows that with dead trypanosomes the sera of 7 of the 8 animals turned positive in the Wassermann and Sachs-Georgi reaction. In most cases the reactions were strong already after 3 to 4 injections. In one case the serum was weak in the Wassermann reaction although distinctly positive with trypanosome extract and in the Sachs-Georgi reaction. In the infected animals the

results were less regular, especially as regards the Wassermann tests, while the lecithin reactions were positive in all cases but one (Table II).

The changes in the reaction during the course of the experiments and also the differences between the various sort of antigens will be referred to later.

TABLE VI.—*Rabbits Injected*

No. of animals		11	19	27
16	No. of days after the first injection	11	19	27
	No. of injections	2	3	4
	Wassermann reaction	vstr,ac,c	0,0,0,0,0,ftr,ac	0,0,0,0,ftr,str,vstr
	Sachs-Georgi with serum diluted 1:2	tr	++	++±
	Complement fixation with trypanosome alcoholic extract	0,w,ac,c	0,0,0,0,w,vstr,ac	0,0,0,0,0,ftr,vstr
17	No. of days after the first injection	11	19	27
	No. of injections	2	3	4
	Wassermann reaction	0,0,w,c	0,0,0,0,0,0,vstr,ac	0,0,0,tr,str,vstr
	Sachs-Georgi with serum diluted 1:2	++±	+++	+++
	Complement fixation with trypanosome alcoholic extract	0,0,w,vstr	0,0,0,0,0,w,ac	0,0,0,0,tr,str
18	No. of days after the first injection	11	19	27
	No. of injections	2	3	4
	Wassermann reaction	d,str,c,c	0,0,0,0,str,c	0,0,0,tr,vstr,c
	Sachs-Georgi with serum diluted 1:2	+	+++	++++
	Complement fixation with trypanosome alcoholic extract	0,tr,vstr,c	0,0,tr,vstr,c	0,0,0,0,d,ac

A second experiment was performed similar to the first.

The results presented in Tables III and IV agree well with the foregoing. In the experiment with dead trypanosomes 7 out of 8 animals showed strongly positive Wassermann reactions after 3 injections corresponding to 6 to 7 mg. dry weight 19 days after the first

injection. All the sera reacted positively with trypanosome extract. Frequently the reactions started suddenly. With lecithin no complement fixation was observed while in the former experiment a few sera gave weak reactions. Weak or moderate flocculation reactions occurred in some instances with lecithin. The Sachs-Georgi tests

with Dead Trypanosomes.

34	40	47	53	62
5	6	7	8	9
0,tr,str,ac,c	w,w,vstr,c	ac,c,c	ac,c,c	d,vstr,ac,c
+++	tr	ftr		ftr
		0,d,str,c	0,d,ac,c	tr,str,vstr,c
34	40	47	53	62
5	6	7	8	9
0,0,w,vstr,c	0,0,w,vstr,c	0,tr,str,ac,c	0,tr,str,ac,c	0,d,vstr,c
++++	++±	++±		++
		0,d,ac,c	0,d,ac,c	0,str,vstr,c

ran parallel to the Wassermann reaction. After 2 injections, 11 days from the beginning of the treatment the fixation tests were already positive in 4 out of 8 animals (see Table III) with Wassermann antigen and 6 were positive with alcoholic trypanosome extract. Presumably the reactions with trypanosome extract precede those

TABLE VII.—*Complement Fixation Tests with*

No. of animals	No. of days after infection........	33	47	57
9	Wassermann reaction...... Complement fixation with extract of trypanosomes..	0,0,0,0,vstr,c tr,d,str,c	0,0,0,0,ac d,tr,w,d	0,0,0,0,0,str,ac,c 0,0,0,w,vstr,ac
	No of days after infection........	33	47	57
12	Wassermann reaction......	0,0,0,ac,c	0,0,str,ac,c	w,str,vstr,c
	No. of days after infection.......	33	47	57
14	Wassermann reaction...... Complement fixation with extract of trypanosomes.	0,0,0,tr,c str,str,vstr,ac,c	0,0,0,0,0 d,ftr,0,tr,str	0,0,0,0,0,0,d,c ftr,0,0,0,vstr,ac
	No. of days after infection........	17	24	32
24	Wassermann reaction......	vstr,c,c	0,0,0,0,tr,vstr,ac	0,0,0,0,0,str,c
	No. of days after infection.......	17	24	32
25	Wassermann reaction......	vstr,c,c	0,0,0,0,ac	0,0,0,ftr,vstr
	No. of days after infection........	9	16	22
27	Wassermann reaction......	w,d,vstr,ac,c	str,vstr,vstr,ac,c	ftr,ftr,d,ac

Sera of Infected Rabbits at Various Stages.

70	84	98	175	
0,0,0,0,0,ac	0,0,ftr,vstr,ac,c	0,0,0,tr,c	w,d,str	
0,tr,vstr,ac,c	tr,w,vstr,c,c	d,w,ac,c	str,c,c	
70	84	98	175	
ftr,tr,str,c	d,w,vstr,c	0,0,w,ac,c	0,0,0	
70				
0,0,0,0,0,0,vstr				
0,0,tr,vstr,c				
37	43	59	70	145
0,0,0,0,tr,vstr,c	0,0,0,0,0,str,ac	0,0,0,vstr,c,c	tr,d,vstr,c	c,c,c
37	43	56	70	145
tr,0,tr,vstr,c	d,ftr,str,ac,c	ac,c,c	c,c,c,c	vstr,ac,ac
36	50	125		
0,0,0,tr,ac,c	0,0,0,0,0,vstr,c	c,c,c		

with the heart extract in analogy to the findings of F. Klopstock. Regarding these reactions some doubt may arise as to whether or not they are due to a fixation of complement with substances derived from rat cells, since the presence of small quantities of these in the injected material could not be avoided. Control experiments with rat blood extracts however seem to rule out the possibility that such an effect influences the results essentially.

Of the infected animals one failed to develop reagins while in another the response was feeble. Again the reactions with lecithin are well pronounced, those with trypanosome extract are weak. In some cases the latter became strong at a later stage (Table VII, Animals 9 and 14). Positive reactions with alcoholic trypanosome extract are reported by other authors in trypanosomiasis. In this and in the first set of infections the sera gave a strong agglutination when mixed with a suspension of live trypanosomes. The phenomenon was not closely examined. (Rieckenberg's reaction?)

For comparison some tests with sera of human and rabbit syphilis are given in Table V.

It may be of interest to give examples of the changes in the reactions occurring during the course of the experiments (Tables VI and VII).

One sees from the tables that the sera of the animals which were continually injected and also in the infected ones, the antibodies tend to decrease or disappear after a certain time. In some of the infected rabbits kept under observation for several months this drop was followed by a recurrence of the reactions (for instance Rabbit 12).

In order to ascertain that the effects described are peculiar to the antigens contained in trypanosomes, control experiments were performed with other materials as, cholera vibrios, emulsions of beef and pig kidney, and with pig serum, the same amounts (in terms of dry weight) being used for the injections as in the experiments with dead trypanosomes. In each case 4 rabbits were taken. Only in 1 animal did a distinctly positive Wassermann reaction develop after 4 injections with beef kidney.

DISCUSSION.

The main result of the experiments described is the fact that the sera of rabbits become strongly Wassermann-positive upon injections of dead trypanosomes. Flocculation reactions according to Sachs-Georgi corresponded to the complement fixation.

The immunization was obtained with small quantities of antigen and almost regularly. In this respect the findings resemble those of F. Klopstock with *Spirochæta pallida*. Although injections with other materials may occasionally give rise to positive reactions,[2] a peculiar quality of the microorganisms studied must be assumed in view of the ease with which they induce the production of the reagins. Equal amounts of other substances as vibrios, organs and serum used in control experiments yielded almost uniformly negative results. Also the difference in the effects of injections of normal and syphilitic organs as demonstrated by Citron and Munk (18) and recently confirmed by Heimann (19) can be explained more readily by the action of the microorganisms as such than by the interpretation favored by Heimann.

As mentioned before Sachs, A. Klopstock and Weil attribute the appearance of the Wassermann reagins to an autoimmunization against tissue lipoids bound to proteins of the spirochetes. A certain difficulty arises for the idea of Sachs and his coworkers from the fact that the method employed in their experiments calls for an intensive treatment with large amounts of antigenic material (serum). Furthermore their findings do not explain the occurrence of the Wassermann reaction just in cases of syphilis in contradistinction to other infectious diseases unless one assume, as the authors do, an exceptional aptitude on the part of the spirochetes to bring forth the antigenic properties of lipoids. Such an assumption however has not yet been substantiated by experimental data. At first sight the demonstration of a particular antigenic activity of the spirochetes and trypanosomes themselves seems to give a more natural answer to the question of the formation of Wassermann reagins.

[2] See Brandt, Guth and Müller (16), van der Scheer (17), Klopstock, F., *Deutsch. med. Woch.*, 1925, li, 1701. In the latter experiments organs were used of rabbits which had undergone a treatment with dead bacilli.

To be sure, there still remain several aspects of the problem requiring further explanation.[3] If the action of the Wassermann-positive sera on organ lipoids is supposed to be a heterogenetic reaction brought about by antibodies for lipoids of the microbes one should expect the reaction with the latter to be more intense or at least nearly equal in strength to the former.   This is true for the experiments of F. Klopstock, and for ours as far as they were performed with injections of killed trypanosomes.   On the other hand the reactions which we obtained with the sera of the infected animals were generally much weaker with trypanosome extracts than with the heart lipoids. While the higher dilution of the trypanosome extract, or the fact that it was not cholesterolized, may possibly account for the weaker reactions it does not explain the difference between the two sorts of experiments, namely infection and immunization with killed microorganisms.   Another discrepancy appears in the tests with lecithin (complement fixation and flocculation).   These are quite generally much stronger in the case of the infected animals.   We found such lecithin reactions also with syphilitic sera of rabbits.   Positive lecithin reactions have been recorded by Sachs as a result of the combination method.

Thus one may conclude that the effects are not altogether conformable in both cases; *viz.*, treatment with dead trypanosomes and infection.

In our opinion, these differences do not disprove the hypothesis that the microbes are the active agent in the formation of the reagins. The substances derived from the trypanosomes during the infection and those formed in the disintegration *in vitro* may be somewhat different and it is not improbable that such variations are reflected in the response of the animal.   Changes in the method of preparing the antigen may suffice to alter the results, and further investigation along this line is desirable.

Still it cannot be stated as yet that the serological changes occurring during the syphilitic infection are due to one single principle. There is for instance not yet a definite explanation for the occur-

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[3] As for instance the observations on flocculation reactions with substances other than lipoids. (*Cf.* Weisbach, W., Wassermannsche Reaktion, etc., Jena, 1924.)

rence of positive Wassermann and flocculation reactions in normal animals.

Mention may also be made of the autohemagglutinins found in infections with protozoa and of autohemolysins (paroxysmal hemoglobinuria), apparently specific (20, 21) for human blood in cases of syphilis, though this phenomenon is rarely met with. Perhaps these observations may be indicative of an autoimmunization.

SUMMARY.

Since it is known that positive Wassermann reactions prevail in trypanosomiasis of rabbits, similar to those in syphilis, trypanosomes were used for an inquiry into the cause of this reaction.

Injections with dead trypanosomes into rabbits proved that these microbes are highly active antigens and suffice in themselves to produce strongly positive Wassermann sera, in analogy to the findings reported by F. Klopstock with *Spirochæta pallida.*

Although a number of questions require further study, yet it seems likely that this antigenic activity of the microbes or their products plays a part in the production of the Wassermann reagins in infections with spirochetes and trypanosomes.

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INFLUENCE OF LIGHT ON THE GROWTH AND MALIG-NANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

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One of the striking features of the disease induced by a certain transplantable neoplasm of the rabbit is the variability of its course and of the general character of its manifestations (1). Not only do individual animals differ in respect to the plane or level of malignancy but well defined group variations are also observed between series of rabbits inoculated at one time and those inoculated at another. On the whole, this variability is seasonal in character in that the disease is most severe in the spring and fall and is usually milder in the summer than in the winter months (2). The influence of the seasonal factor has been interpreted as operating upon or affecting general animal economy and susceptibility or resistance to disease is considered as a functional activity of the animal organism.

Although many external and internal factors undoubtedly participate in the seasonal variability of this malignant disease, there appears to be a correlation between sunshine and the manifestations and outcome of the condition. That is, the actual hours of sunshine, together with the rate, extent and persistence of change in the curve of sunshine over a given period, can be related to the observed general level of malignancy. The experiments upon which this relationship has been based were carried out with rabbits kept in rooms which receive practically all light diffused through ordinary window glass so that the effects observed cannot be wholly attributed to the shorter ultra-violet rays.

The idea of a correlation between the external factor of light on the one hand, and the physical state and functional activities of the animal organism on the other, has been tested by a series of experiments in which conditions of light could be controlled. The particular

points studied in beginning the work were the effects produced by a continuous illumination of maximum intensity practicable with simple equipment and by an absence of light, first upon the physical state of normal rabbits as shown by the weights of organs (3) and second, upon the course and character of the malignant disease. The results of the first tumor experiment which were summarized in a preliminary note (4) published soon after the conclusion of the work are here reported in full.

Methods and Material.

The experiment was begun on January 16 and concluded on April 15, 1925. Three animal rooms, with similar environmental conditions other than those of lighting, were employed. These rooms will be referred to as the light, the dark and the control or unaltered room, respectively.

A *constant* source of light was furnished by twelve 1000 watt Mazda lamps and three Cooper Hewitt 50 inch low pressure mercury arcs, Type P, in crown glass, arranged on a rectangular frame in the center of a room from which all other light was excluded. The lamps and arcs were distributed in three horizontal rows 2½ feet apart and each row contained four lamps and one arc. The animal cages were placed parallel with and on either side of the light source at a distance of 3¼ feet.

The intensity of light reaching the cages was not entirely uniform, but on the average was 425 foot candles. The spectrogram of the mercury arc furnished by the Cooper Hewitt Company, shows that the crown glass absorbs all light below a wave-length of 3022-28 Ångström units while that of the Mazda lamps is cut off at about 3100 Ångström units. Our primary object in so far as the quality of the light was concerned, was the absence of rays which are absorbed by ordinary glass, as in the case of the diffused sunlight of the laboratory.

A second room was arranged so that all light was constantly excluded except during the time the animals were fed or examined, when a single 30 watt Mazda lamp was employed. This light was not used for more than 1 hour per day.

An ordinary animal room which has a southern exposure was used as the control room. Practically all the light of this room was diffused sunshine which passed through the glass of two large windows.

The temperature of the three rooms was maintained at 70° to 75°F. by automatic regulation of the common heating and ventilating systems. In the case of the light room, this was supplemented by an electric fan intake of outside air which also served as an aid to satisfactory ventilation. With this equipment, it was not possible to keep the temperature of the light room within the above limits during the warmer months, so that in subsequent experiments to be reported later, the

type of illumination was modified. The humidity of the three rooms varied with that of the outside air.

The rabbits employed were representative of the usual breeds and types commonly used in this laboratory. They were adult male animals, approximately 1 year old, and were assembled from a carefully selected stock 1 week prior to the experiment. During the entire period of observation, each rabbit was separately caged and fed the standard diet of oats, hay and cabbage.

On January 16, 1925, 10 rabbits were placed in each of the three rooms where they remained for the duration of the experiment. On February 16, 1 month after they had been living in an environment of constant light or of constant darkness or in the variable diffused sunlight of the laboratory, each rabbit was inoculated in the right testicle with 0.3 cc. of a tumor emulsion prepared from an actively growing primary tumor. This neoplasm is considered to be of epithelial origin (1). The experiment was terminated on April 15, 2 months after inoculation, at which time all surviving animals were killed by an injection of air into the marginal ear vein. This period was selected upon the basis of previous experience as being sufficiently long to include the majority of deaths due to tumor involvement or to permit recovery to take place.

Detailed records including body weight determinations were kept covering the clinical course of the disease and each animal received a careful postmortem examination with special reference to the presence or absence of metastases, the number and distribution of metastases, the degree of involvement of different organs and the state of the growth. The distribution of metastatic foci in particular has been used as a basis for estimating the general character of the disease in those cases in which secondary growths were found. The method used involves the calculation of percentage values of the number of tumor sites or foci in terms of the number of theoretical foci as shown by the actual location of metastases in the first twenty generations of tumor animals (5). There are certain obvious objections to this method. For instance, such organs as the liver or kidneys may be markedly involved with numerous tumors or by only a few which destroy little tissue. However, a comparative classification of the disease, whether of high, moderate or low malignancy, may be brought out in percentage terms by an arrangement of metastatic distribution according to the following divisions:

I. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system..30 possible foci
II. Lungs and pleura, liver, kidneys and pancreas............5   "   "
III. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum...19   "   "
IV. Suprarenals and eyes................................... 4   "   "

This system of grouping metastatic foci was selected for these reasons:

It has been found, from the study of several hundred rabbits inoculated in the testicle with the tumor, that there is usually a widespread distribution of metastases, extensions and implantations in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation   In these instances, tumors may be found in many organs and tissues as in the skin, the superficial lymph nodes, the muscles, the bones and bone marrow, the heart, the spleen, and the endocrine glands as well as in the liver, the kidneys, the lungs, the retroperitoneal and mediastinal tissues and the serous membranes of the abdominal and pleural cavities   In instances of a somewhat less malignant disease, the most conspicuous and frequent metastases are found in the liver, kidneys, lungs and pancreas. A level of still lower malignancy is chiefly characterized by the predominance of extensions of tumor from the primary growth in the testicle to the retroperitoneal and mediastinal tissues and by implantations upon the omentum, mesentery and parietal peritoneum. If death occurs in these animals during the first 2 months after inoculation, the extensions and implantations referred to are found to be of an extreme grade, or, more frequently, some organ such as the kidneys or hypophysis is also involved. Finally, in those animals in which the disease is very mild, metastases may be found only in such sites as the eyes or suprarenal glands which of course are also involved in instances of high malignancy. On the other hand, metastases to the skin, muscles, bones and endocrine gland group practically never occur in cases of low malignancy.

Results.

The results of this experiment consist, first, of the clinical observations in which the general physical condition of the animals, the character of the primary growth and the development of metastases recognizable during life, are of especial interest; second, the mortality rate including the time of death with reference to the time of inoculation; and third, the postmortem observations, particularly as regards the condition of the primary tumor, the animal incidence of metastases and the number, distribution and state of these growths.

As far as could be determined by observation and by body weight, the health of the rabbits kept under the conditions of constant illumination was excellent, and while no outspoken deleterious effects were observed in the animals kept in the dark, there were indications furnished by body weight determinations to the effect that this environment was a less favorable one (Table I). Only the weights of rabbits surviving the experiment have been used in this connection since a loss of weight together with other manifestations of cachexia

frequently occur in association with a widespread tumor growth in animals which succumb to the disease. Reference to the table shows that the groups of rabbits kept in the environment of constant light and of the variable sunlight conditions of an ordinary animal room for 3 months showed a well marked increase in body weight while the group in constant darkness gained comparatively little. Reckoning from the mean weight on admission the controls gained more than the light group due, probably, to the lower initial figure of the controls. However, the final mean weights of both groups were the same. The net gains over the first observations available after the rabbits from the light and dark rooms had been placed in these rooms were practically the same for the light group and the controls, but the dark room group gained only one-third as much as the others.

TABLE I.

Changes in Mean Body Weight.

Group	On admission	Experimental period			Percentage gain	
		1 wk.	4 wks. Day of inoculation	12 wks.	Over admission weight	Over first experimental weight
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
Controls........	2120	2350	2465	2660	25.5	13.2
Lights..........	2264	2342	2428	2675	18.2	14.2
Darks..........	2283	2238	2288	2346	2.7	4.8

A primary tumor developed in each rabbit of the experiment. In the control group,[1] there was a rapid and extensive primary growth for the first 3 weeks, after which time regression took place in the 5 rabbits which survived the experimental period of 2 months, and in 3 of these complete absorption with healing occurred. In the 4 rabbits dying from tumor, there was relatively little necrosis of the primary growth. In the rabbits from the dark room the primary growth was very rapid in 3 animals which early succumbed to the disease, while in 7 rabbits it grew more slowly and ultimately regressed and at the end of the experiment it was entirely necrotic in 2 and

[1] One of the control rabbits died of an intercurrent infection so that this group consists of 9 animals.

healed in 5 instances.   In the group exposed to constant illumination, the initial growth of the tumor in the majority of the animals was unusually rapid and extensive but it eventually became entirely necrotic in 5 and completely healed in 4 rabbits.   In the remaining animal, which finally succumbed to the disease, approximately one-half of the primary tumor was living.

TABLE II.

Analysis of Results.

Group	No. of rabbits	Metastases found during life		Mortality			Postmortem observations of metastatic foci						Actual and probable recoveries
		Animal incidence	Total No.	No.	Rate	Time after inoculation	Animal incidence	Total No.	Relative rate	Actual rate	No. of foci in		
											Deaths	Survivors	
		per cent			*per cent*	*wks.*	*per cent*						*per cent*
Controls...	9*	44.4	10	4	44.4	4,4, 4.5, 4.5	56.0	81	9.0	16.2	30, 17, 15, 15	4 in 1 rabbit 0 in 4 rabbits	55.6
Darks......	10	30.0	7	3	30.0	3.5, 3.5, 4	40.0	69	6.9	17.3	26, 25, 17	1 in 1 rabbit 0 in 6 rabbits	70.0
Lights.....	10	20.0	6**	1	10.0	6.5	50.0	27	2.7	5.4	22	5†in 4 rabbits 0 in 5 rabbits	90.0

   * 1 rabbit of the original 10 died of an intercurrent bacterial infection during the experiment.
   **Two of these metastases eventually became completely healed.
   † Three of these metastases occurring in 2 rabbits were small and entirely necrotic.

Metastases in superficial parts of the body which were recognized during life developed in several rabbits (Table II).   In the control group there were ten such secondary growths in the eyes, superficial lymph nodes, subcutaneous tissue and bones distributed among 4 rabbits—an animal incidence of 44.4 per cent.   In the group kept under conditions of constant darkness, the animal incidence of metas-

tases detected clinically was 30.0 per cent; seven tumors were found in the eyes, lymph nodes, skin and subcutaneous tissue. On the other hand, in only 2 rabbits of the light group—20.0 per cent—were such growths recognized. In 1 rabbit the unusual occurrence of regression and ultimate healing of eye metastases was observed while in another, four tumors were found in the muscles, bones, subcutaneous tissues and superficial lymph nodes.

The mortality rates of the three groups were—controls 44.4 per cent; darks 30.0 per cent; lights 10.0 per cent (Table II). Among the controls, 4 rabbits were killed, 4, 4½ and 5½ weeks after inoculation because of the development of paralysis or other symptoms indicative of impending death. A similar procedure was necessary in three instances in the dark room group, 3½ and 4 weeks after inoculation. Among the rabbits in the light room, on the other hand, there was but one such instance. The symptoms which necessitated the sacrificing of this animal 6½ weeks after inoculation, developed much later and more slowly than those of similar cases in either the control or dark room groups.

At postmortem examination, it was found that the incidence of metastases was approximately the same in all three groups, but there were distinct group differences in the number of foci[2] (Table II). Among the 9 control rabbits there were 81 foci of metastatic growths, or 9 per animal, 69 foci in the dark group, or 6.9 per animal, and only 27 foci in the light group, or 2.7 per animal. A comparison of actual metastatic rates in which only rabbits with secondary growths are considered, shows that there was little difference between the control and dark groups, but in the case of the light group the rate was approximately one-third those of the others.

The number of metastatic foci in individual rabbits is given in Table II. In the fatal cases, the largest number occurred in a con-

[2] It should be pointed out that the figures refer to the number of organs or tissues involved, and not to the numbers of secondary growths, and consequently the expressions "foci of metastases," "distribution of metastases," or "metastatic rate" are used rather than "number of metastases." On the other hand, the figures for clinical metastases detected during the life of the animal refer to the actual number of individual secondary tumors found. The present basis of counting foci of metastases is slightly different from that employed in the preliminary note (4).

trol animal in which 30 foci were counted and in the others, there
were 15 to 26.   But in so far as the severity of the disease may be

Distribution of metastatic foci
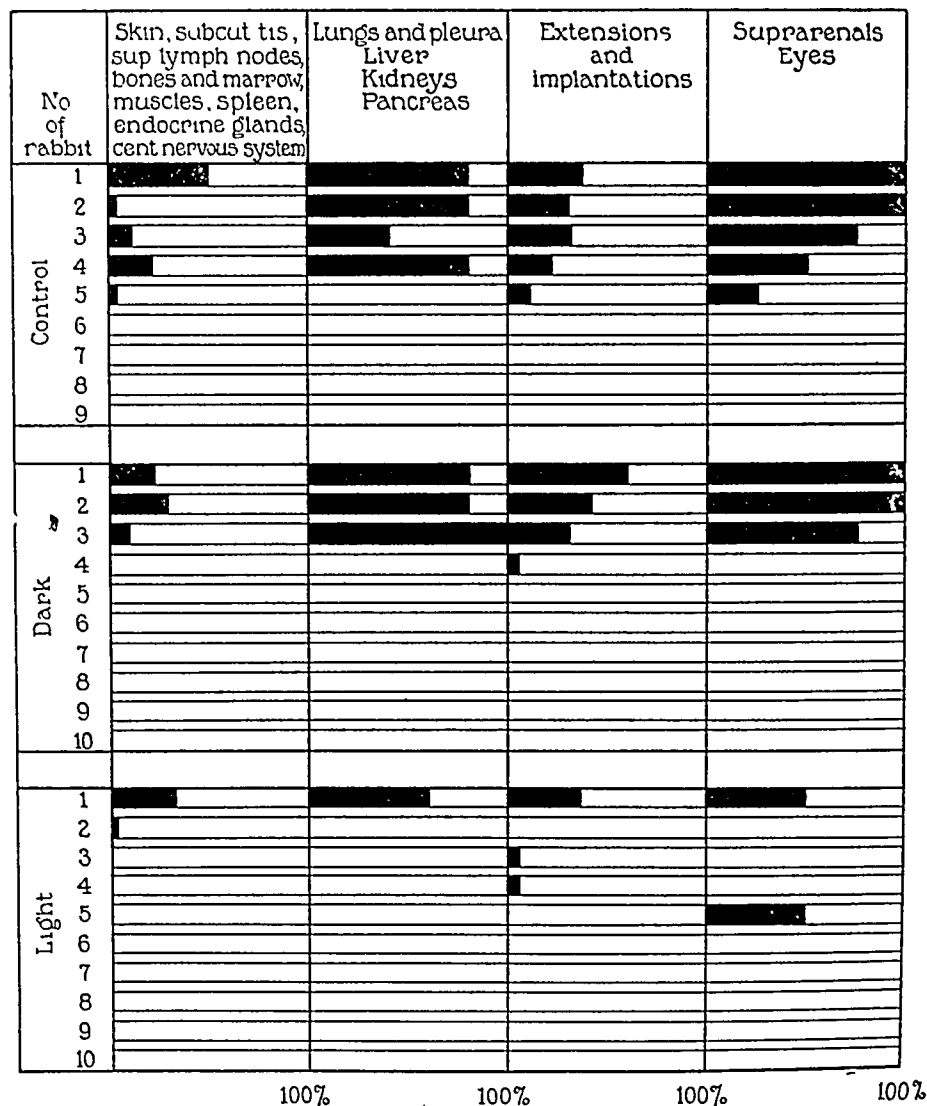
TEXT-FIG. 1. Distribution of metastatic foci.,

judged from the mean number of metastatic foci, there was practi-
cally no difference in the three groups—(controls 19.25, darks 22.7,

lights 22.0). Among the rabbits which survived the experimental period of 2 months, postmortem examination revealed 4 metastatic foci in one control, a single focus in a dark room rabbit and 5 foci distributed among 4 light room animals. In 2 of the rabbits from the light room, the three secondary growths found were small and entirely necrotic. It is probable that none of the 6 surviving rabbits with metastatic foci would have died from the effects of tumor growth.

The character of the disease which developed in the three groups may be compared upon still another, and in many respects a more impressive basis, namely, the distribution of metastatic foci, as is illustrated by Text-fig. 1. In this graph, each rabbit is represented by a horizontal column, divided into four sections corresponding to the four general divisions of secondary growths previously described, while the shading of the sections represents the metastatic rate in percentage terms of the possible number of foci.

This diagrammatic representation illustrates in a striking manner the low plane of malignancy of the disease in the light room group. The metastatic rate in all four divisions is seen to be much lower than that of either the controls or the dark room groups. As far as the dark room series is concerned, the metastatic rate is, on the whole, somewhat lower than that of the controls if one takes into consideration the number of animals with secondary growths and the degree of involvement of the two divisions of foci which are predominately affected in instances of severe or well marked malignancy, namely, the division which includes the skin, muscles, bones and glands of internal secretion, and that of the lungs, liver and kidneys. In the case of the light room group, there was but 1 rabbit in which a disease characterized by a widespread distribution of metastatic growths developed while there were 3 such instances among the dark room and 4 among the control animals. In a 5th control rabbit, there was a wider distribution of tumor than in a 4th dark or in 4 other light room rabbits in which metastases were found and in 2 of these last animals, it will be recalled that the secondary growths were entirely necrotic. Finally, there were 4 controls as contrasted with 6 dark and 5 light room animals in which no metastases were found.

DISCUSSION AND CONCLUSIONS.

This experiment was undertaken for the purpose of obtaining information on the influence of light on the character of disease in rabbits induced by a transplantable malignant neoplasm. To this end, the attempt was made to insure conditions which would be common to all animals of the experiment with the exception of the light environment. Comparable conditions of temperature and humidity prevailed in the three rooms in which the experiment was carried out, all the rabbits were fed the same diet, and the animals themselves, assembled at the laboratory at the same time, were well matched as to breed and age. Individual animal variation could not, of course, be wholly eliminated, but this factor was controlled by the selection and number of rabbits employed in each group.

The lighting of the three rooms was the variable factor of the experiment. One group of rabbits, kept in a well lighted animal room was exposed to the daily fluctuations of sunshine diffused through ordinary window glass. Another group was placed in a room which was continuously and constantly lighted by Mazda lamps and mercury arcs in crown glass. The third group was kept in a room from which all light was constantly excluded except for very brief daily periods.

The clinical and postmortem observations of the three groups of rabbits inoculated with the malignant tumor and kept in these different surroundings bring out distinct variations in the course and character of the tumor process. The most highly malignant disease occurred in the control group living under ordinary indoor daylight conditions. The mortality rate was the highest, the incidence of secondary growths detected during life or found at postmortem examination was also the highest and there was the widest distribution of metastatic foci. In contrast to these effects was the extremely mild disease which developed in the rabbits kept under conditions of constant illumination. The mortality rate of this group was only one-fourth that of the controls and the duration of life of the single rabbit which succumbed to the disease was considerably longer than that of comparable controls. In addition, there was a lower incidence and a smaller number of metastases found during life together with a smaller number of metastatic foci observed at autopsy.

The disease which developed in the group living in practically constant darkness was also not as severe as that of the controls but was considerably more malignant than that of the light room group. As compared with the controls, the mortality rate was slightly lower, the incidence and number of clinical metastases were somewhat less, and there were fewer sites of secondary growths as revealed by postmortem examination. In addition, a higher percentage of rabbits either recovered wholly or was in process of recovery. Although the effect of constant light exclusion was much less pronounced than that of constant and continuous illumination, it appeared to be in the same direction of diminished malignancy.

There were certain special features of the disease that developed in the rabbits kept in the light and dark rooms which have a direct bearing on the influence associated with these environments. The course and character of the primary tumor in the control and dark room groups were in general accord with what is usually observed in the majority of normal rabbits. That is, the tumor underwent a rapid growth during the first 2 weeks and, in animals succumbing to the disease 3 to 5 weeks after inoculation, showed little tendency toward regression while in most rabbits surviving as long as 2 months, it was found to be wholly or largely necrotic or completely healed. In contrast with the control and dark room groups, the initial growth of the primary tumor in the majority of the light room rabbits was unusually rapid and extensive so that the first impression of the disease was one of heightened severity. The subsequent course of the primary growth, however, was in agreement with the low level of malignancy shown by the disease of this group, for it became entirely necrotic or became completely healed in all but 1 rabbit.

Although there was practically no difference in the incidence of metastases in the control and light room groups, the greatly lessened number and the markedly restricted distribution of the growths in the rabbits in the light room, indicate that these animals were able to restrain or inhibit the development of the majority of metastatic foci. This finding is further supported first, by the unusual occurrence in 1 rabbit of the complete healing of the metastatic growths in the iris of both eyes; second, by the postmortem finding in 2 other rabbits of but 3 small and entirely necrotic metastatic tumors and

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finally by the comparative lateness with which the terminal stages developed in the 1 rabbit of the group which eventually succumbed to the disease.

The incidence of metastases in the dark room group was slightly lower than in the others. The secondary growths were confined almost wholly to 3 rabbits in which the disease exhibited well marked malignant characteristics, while in the other animals it was extremely mild. These results indicate first, that constant darkness has a slight but definite effect upon the course and character of the disease and second, that this effect differs in some respects from that observed under conditions of constant illumination. It would appear that under the influence of the conditions obtaining in the dark room, those rabbits which possessed a high resistance to tumor growth, either natural or acquired, were able to exercise this power more efficiently but that the influence was not sufficient to enable less resistant animals to deal successfully with the disease. On the other hand, the restraining or inhibitive effect associated with constant illumination was, to a greater or less degree, seen in all rabbits of the group.

The results of this experiment are interpreted as furnishing experimental evidence in support of the idea referred to in the beginning of this paper, that there is a relation between the factor of light and the manifestations and outcome of the malignant disease with which we have been working. It will be recalled that, in general, the periods of maximum and minimum sunlight, corresponding with summer and winter were associated with relatively low levels of malignancy while the periods of greatest malignancy occurred at times of abrupt and rapid changes in the hours of sunshine, coinciding roughly with the spring and autumn months.

In the experiment here reported, the most malignant disease developed in the group of rabbits living in an ordinary room, exposed to the influence of the variations in sunlight, passing through ordinary glass, that prevailed during these months (February, March, April). In contrast with these results, a disease of low malignancy occurred in the group kept in an environment of constant and intense illumination. Furthermore, the plane of malignancy of the disease in the rabbits living in a room from which almost all light was excluded was

found to be somewhat lower than that of the controls although it did not approach the low level of the group exposed to constant illumination. It would appear, therefore, that the conditions of light in the experiment reported were associated with variations in the course and character of the malignant disease comparable with those of a seasonal nature which normally occur throughout the year.

The mechanism by which these effects are induced is not known. We have considered the influence of the seasonal factor in disease as operating upon or affecting general animal economy and susceptibility or resistance as a functional activity of the animal organism. Hence it appears that resistance to tumor growth as displayed by rabbits living under conditions of constant intense illumination and, to a less extent, in almost constant darkness is more potent or efficacious than it is in the case of rabbits exposed to the daily fluctuations of diffused sunlight.

Finally, it should be stated that no attempt was made in this experiment to produce ideal conditions for diminishing or enhancing tumor malignancy. The purpose was to determine whether variations in the course and character of the malignant disease took place, first, under conditions of constant and continuous illumination in which none of the shorter ultra-violet rays participated and, second, under conditions of constant darkness.

SUMMARY.

An experiment is reported in which an environment of constant and continuous light excluding the shorter ultra-violet rays, and one of constant darkness, have influenced the course and character of a malignant disease of rabbits induced by a transplantable neoplasm.

Under the influence of constant light the level of malignancy was observed to be low; under the influence of constant darkness the level of malignancy was somewhat lower than in the control animals living under ordinary indoor light conditions, but the level was not as low as among the animals constantly illuminated.

These observations furnish experimental evidence in support of the idea that there is a correlation between the external factor of light on the one hand and the manifestations of an experimental malignant disease on the other.

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THE INFLUENCE OF LIGHT ON THE REACTION TO INFECTION IN EXPERIMENTAL SYPHILIS.

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The occurrence of wide variations in the character and course of the disease produced in rabbits by inoculation with a given strain of *Treponema pallidum* was recognized long since by us as one of the outstanding features of the experimental infection and we have emphasized the fact that the more decided variations show a tendency to a seasonal periodicity with the occurrence of periods of maximum severity at some time during the spring and fall (1, 2). A survey of available data bearing on this subject seemed to indicate a relation between the course of infection and prevailing meteorological conditions (3). This was particularly true of sunlight expressed in terms of the hours of sunshine over a given period of time. But, the data at hand were not sufficient to indicate the precise nature of the relation that obtained or to warrant a definite conclusion as to the relative importance of the several factors that might be concerned in the production of these effects. It seemed, however, that we had to deal with a general rather than a specific problem, or, that the effects noted in the case of syphilis were incidental to the occurrence of profound changes in the animal organism, influenced in some way by physical environment (3).

With a view to obtaining further information on this subject, several lines of investigations were undertaken. One of these was a systematic study of the normal animal organism in relation to physical environment; another assumed the direction of consecutive observations on the course of syphilitic infections in comparable groups of animals over long periods of time, while a third series of investigations was concerned with direct tests of the effects of physical influences on the reaction to infection. Some of the results of these investigations

have been reported (4, 5). *The object of the present paper is to* report the results of a series of experiments dealing with the influence of light on the reaction to syphilitic infection. These experiments were undertaken for the purpose of determining, first, whether the course of disease could be influenced by variations in the light environment of animals, comparable to those that occur in consequence of changes in season or weather, and, second, something as to the nature of the effects that might be produced by changes in environment or conditions of this general order. We were, of course, concerned with conditions that might prove to be favorable or unfavorable, but in this instance no effort was made to adjust experimental conditions from this point of view lest by so doing, we might defeat the main object of the experiments. The indications were that at the outset extreme conditions should be employed.

EXPERIMENTAL.

Methods and Material.

The results to be reported represent a comparison of the course of infection in three groups of rabbits living under conditions which, as far as possible, differed only with respect to their light environment. One group of animals, which may be designated as the controls, was kept in a well lighted, well ventilated room with a southern exposure. The temperature of the room was automatically regulated and maintained at a level of 70° to 75°F. while the humidity varied with that of the outside air. The light received by these animals was sunlight which for the most part was filtered through window glass; the amount and quality of the light were determined by natural circumstances.

A second group of rabbits was kept in a room from which all sunlight was excluded; otherwise, the conditions were the same as those of the first room. These animals lived in total darkness except for a brief period each day when it was necessary to use a small Mazda lamp while cleaning cages, feeding, and carrying out routine examinations. For convenience these animals will be referred to as the dark group.

The third group of animals, which may be designated as the light group, was kept in a room similar to the others with sunlight excluded. This room was lighted continuously by 13 Cooper Hewitt, low pressure, mercury arc lights in crown glass (Type P, 50 inches long). The light supplied by these lamps has a wave-length ranging from 3022 to 5790 Ångstrom units with the greatest concentration between 3650 and 5790. The lamps were mounted on a rectangular frame extending down the middle of the room and were distributed so as to equalize the light which reached cages in a plane parallel with the frame on each side of the

room at a distance of approximately $3\frac{1}{2}$ feet. With this arrangement of lights, an illumination was maintained which amounted on an average to 200 foot candles, being slightly higher opposite the center of the frame than elsewhere. To insure proper ventilation and as an aid in controlling the temperature, a special system of ventilation was used in this room.

The conditions compared were, therefore, (1) intermittent and irregular exposure to diffuse, filtered sunlight of variable quality and intensity, (2) complete exclusion of light, and (3) continuous exposure to artificial light of a given quality and intensity.

Three experiments were carried out for the purpose of determining the effects of these conditions on the reaction of rabbits to syphilitic infection. In each case, 30 male rabbits were used. They were placed in individual cages and divided into three groups of 10 animals each, as nearly comparable as possible with respect to age, breed, and weight. The stipulated conditions of the experiments differed in only one respect, namely, the length of time animals were kept under the conditions described prior to inoculation. In the first experiment, the period was 2 weeks, in the second 4 weeks, and in the third 6 weeks. There were, in addition, differences referable to conditions that prevailed at the time successive experiments were carried out. These are of especial interest in connection with the results obtained in control animals. It is sufficient to say that the first group of animals was inoculated in October, the second in November, and the third in December, 1925. The period covered by the three experiments was, therefore, from October 1, 1925, to April 9, 1926.

All animals were inoculated in one testicle with the Nichols strain of *Treponema pallidum*. The emulsions used were prepared from actively growing testicular lesions and contained from 1 to 3 spirochetes to the microscopic field. Each animal received 0.2 to 0.3 cc. of the emulsion, the exact amount depending upon the spirochetal content. Animals in a given experiment received equal amounts of the same emulsion and the order of inoculation was the same in all cases, namely, controls, light, dark.

The period of observation following inoculation was 4 months, giving a total period of observation of $4\frac{1}{2}$, 5, and $5\frac{1}{2}$ months respectively for the first, second, and third experiments.

In these experiments, special attention was given to the time and frequency of occurrence and to the duration of successive phases of the reaction to infection with a view to reducing the comparison of results to as accurate a basis of quantitative measurement as possible. The particular conditions chosen for comparison were, (1) the incubation period of primary lesions, (2) the time and frequency of occurrence of a critical edema in the inoculated testicle, (3) the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), (4) the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones, or eyes, (5) the number of foci affected by such lesions, and (6) the proportion of animals that showed complete healing of all lesions during the period of observation (4 months).

For the most part, the methods employed in recording results (Tables I and II and Text-figs. 1 and 2) require no explanation. The term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparisons of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

In Table I and Text-figs. 1 and 2 the results for focal distribution are given in absolute numbers; all other results are in per cent.

Results.

The results of the experiments are recorded in Tables I and II, and Text-figs. 1 and 2. It will be noted that the figures given in Tables I and II represent group values; the detailed results are given in Text-fig. 1 which shows the entire sequence of events from the occurrence of the first primary lesion to the development of the last generalized lesion.

TABLE I.

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions Actual (F. D. Act.) and Relative (F. D. Rel.).

Exp.		Primary lesions	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					Incidence	Focal distribution	Focal distribution
		per cent	per cent	per cent	per cent	actual	relative
I	C.	100.0	40.0	100.0	80.0	8.0	6.4
	D.	100.0	60.0	100.0	100.0	5.3	5.3
	L.	100.0	90.0	80.0	70.0	3.6	2.5
II	C.	100.0	30.0	100.0	70.0	4.1	2.9
	D.	100.0	70.0	90.0	80.0	5.0	4.0
	L.*	100.0	77.7	88.8	77.7	2.3	1.6
III	C.	100.0	40.0	100.0	70.0	4.1	2.9
	D.	100.0	70.0	100.0	100.0	5.3	5.3
	L.	100.0	80.0	70.0	50.0	4.8	2.4
Mean values	C.	100.0	36.6	100.0	73.3	5.4	4.1
	D.	100.0	66.6	96.6	93.3	5.2	4.9
	L.	100.0	82.5	79.6	65.9	3.6	2.2

* One animal in this group died at the beginning of the experiment; there was also 1 death in the control group and 1 in the dark at the termination of the first experiment.

TABLE II.

Mean Time of Occurrence of Various Phenomena of Infection Estimated in Days from the Date of Inoculation.

Exp.		Incubation	Edema	Metastatic orchitis	Generalized lesions		
					First	Last	Mean of all
		days	days	days	days	days	days
I	C.	12.6	21.5	41.0	54.9	82.4	65.5
	D.	12.4	26.6	42.7	53.9	76.4	60.4
	L.	13.0	27.2	36.4	58.8	73.4	65.2
II	C.	16.1	33.7	45.8	55.0	70.3	69.4
	D.	12.8	30.0	46.6	59.5	84.1	67.1
	L.	14.4	34.6	46.3	63.9	66.4	63.1
III	C.	18.1	28.8	39.6	63.7	72.4	65.0
	D.	11.3	29.3	45.3	53.1	72.4	61.1
	L.	15.2	25.4	50.1	56.2	65.0	61.1
Mean values	C.	15.6	28.0	42.1	57.9	75.0	66.6
	D.	12.2	28.6	44.9	55.5	77.6	62.9
	L.	14.2	29.1	44.2	59.6	68.3	63.1

TEXT-FIG. 1.

Incidence and focal distribution of generalized lesions
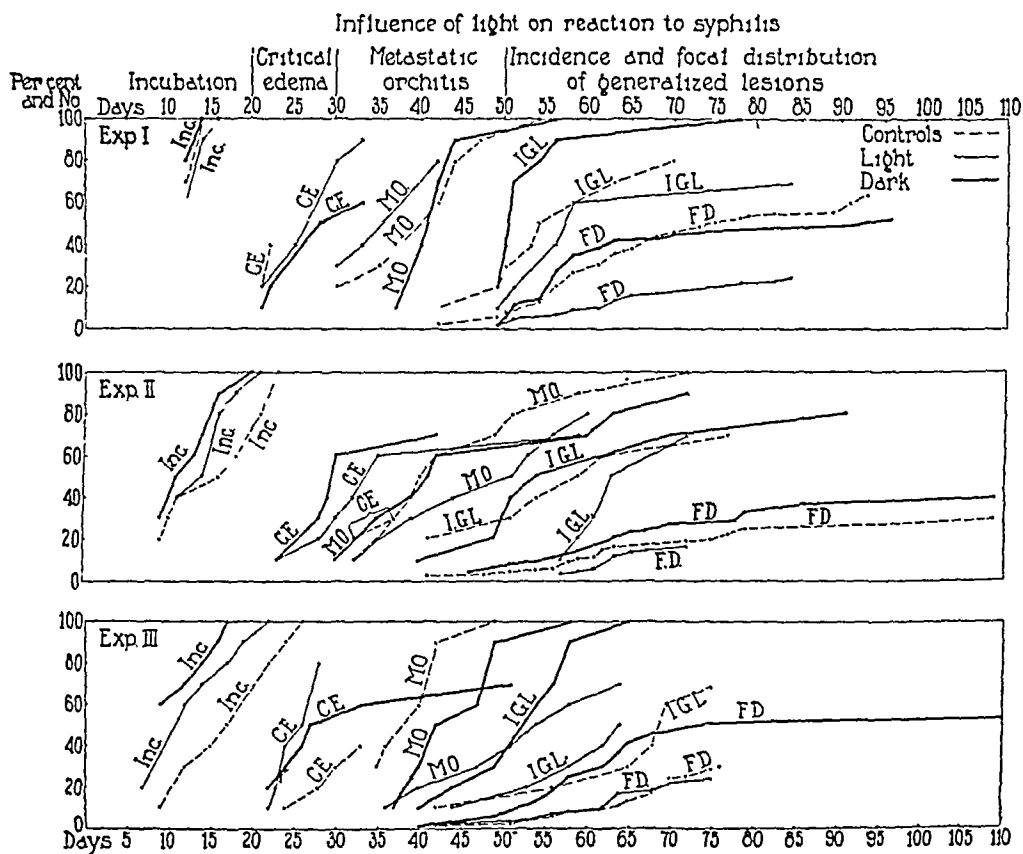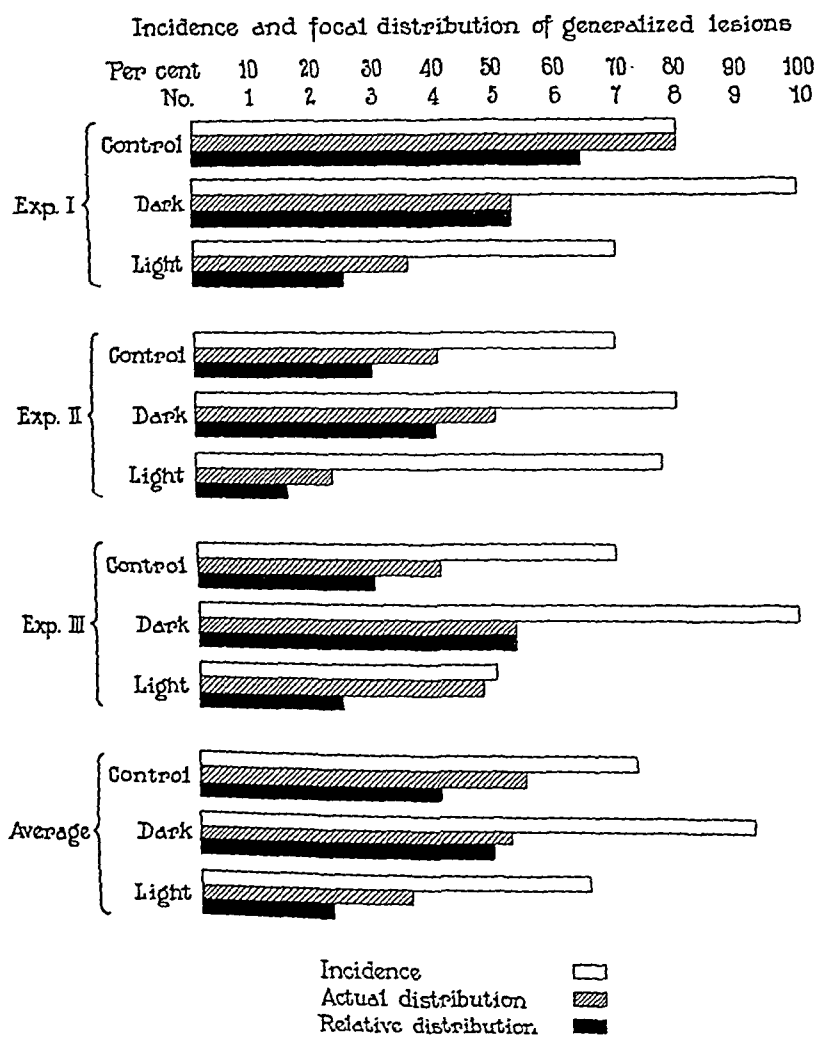
TEXT-FIG. 2.

DISCUSSION AND CONCLUSIONS.

In analyzing the results of the experiments, the most important point to be determined is whether the light environment of the three groups of animals produced any significant difference in their reaction to infection.   The effect of any particular condition on the course of disease is of subsidiary importance.   The simplest method of approaching this problem is by a comparison of the results obtained for various reactive phenomena in the order of their occurrence, bearing in mind the relations that obtain and the general principles that govern the evolution of syphilitic infections (6).[1]   Moreover, the discussion may be limited almost entirely to a consideration of values obtained for group means as the results given by a more detailed analysis of the behavior of individual animals or a consideration of the character of the lesions produced lead to exactly the same conclusions.

Incidence of Primary Lesions and Length of Incubation Period.— All animals in these experiments developed primary lesions but there were some suggestive differences in the incubation period.   In the first experiment (Table II, and Text-fig. 1), the incubation period was essentially the same for all three groups of animals with the controls occupying a position intermediate between those in the light and those in the dark.   In the second and third experiments, the controls showed a progressive prolongation of the incubation period which amounted to approximately 40 per cent.   The animals exposed to artificial light displayed a similar tendency but the differences were less.   In the case of the animals in the dark, the incubation period was remarkably constant and such differences as occurred were in the direction of a reduction rather than a prolongation of the time. Combined results for the three experiments, with an average of 15.6 days for the controls, 14.2 for the light, and 12.2 days for the dark group, give a fair index of the promptness of the primary reaction to infection in the three groups of animals.

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[1] In addition to the relations that are discussed in the paper referred to (6), it should be noted that there is a basic tendency to the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits.   With highly virulent strains of *Treponema pallidum* the interval, or reaction time, is approximately 2 weeks and any decided departure from this basic value may be regarded as significant.

Critical Edema.—The occurrence of a critical edema in an inoculated testicle or in association with any other syphilitic lesion is a very variable but highly significant phenomenon. It is indicative of an intense reaction and usually marks the termination of a local reaction, either temporary or final (7).

In the present instance, there was a very striking and constant difference in the frequency with which edema occurred in the three groups of rabbits. The differences are well represented by the combined results for the three experiments which give 36.6, 66.6, and 82.5 per cent respectively for the control, dark, and light groups.

The figures given in Table II indicate a closer agreement with respect to the time at which the edema occurred. In the case of both the control and light groups, the interval between inoculation and the development of edema varied considerably from one experiment to another but remained fairly constant for animals in the dark. If, for the purpose of measuring the progress of the reaction, the time, in days, is estimated from the appearance of the primary lesions, instead of the date of inoculation, the results are slightly different:

	Control	Dark	Light
Exp. I......................	8.9	14.2	14.2
" II......................	17.6	17.2	20.2
" III......................	10.7	18.0	10.2
Mean......................	12.4	16.5	14.9

Still, these figures, as well as those given in Table II, are somewhat misleading. By reference to Text-fig. 1, it will be seen that, with the the exception of 1 animal each in the light and dark groups of the second experiment and 1 in the dark group of the third experiment, the time of occurrence of edema in individual animals was fairly uniform. The delay in the occurrence of the reaction in the 3 animals referred to overshadows the group tendency so that if the figures are corrected by the omission of these animals, we obtain the following results:

From Inoculation.

	Control	Dark	Light
Exp. I......................	21.5	26.6	27.2
" II......................	33.7	26.0	28.5
" III......................	28.8	25.5	25.4
Mean.....................	28.0	26.0	27.0

From Incubation.

	Control	Dark	Light
Exp. I......................	8.9	14.2	14.2
" II......................	17.6	13.2	14.1
" III......................	10.7	14.2	10.3
Mean.....................	12.4	13.9	12.9

A comparison of results on this basis shows a remarkable constancy in the occurrence of edema among the animals in the light, as well as those in the dark, with a possible tendency toward a shortening of the reaction time. The differences in mean time for the three experiments are comparatively slight, but they are of interest as measures of the progress of the reaction to infection. For example, it will be noted that the time from inoculation to the occurrence of edema in the animals in the dark was shorter than in the controls or the light group, due largely to differences in the incubation period, whereas the time from the development of primary lesions was longer. These differences are suggestive of differences in the efficiency of the reactive mechanism which become more apparent in subsequent reactions.

The most important points brought out by the analysis of the results with respect to the occurrence of a critical edema, are, therefore, the high incidence of edema and the constant time relations shown by both the animals in the dark and those in the light as compared with the much lower incidence and irregular reaction time of the controls.

Metastatic Orchitis.—The development of lesions in an uninoculated testicle occurs with such regularity in rabbits infected with virulent strains of *Treponema pallidum* that the failure of such lesions to occur

may be regarded as evidence of an unusually effective suppression of the infection by some preceding reaction. Variations in the time of development of metastatic lesions are apt to occur and in general the time of occurrence bears a direct relation to the efficiency of preceding reactions.

The outstanding feature of the results as regards the occurrence of metastatic orchitis is the diminished frequency of such lesions in the light animals as compared with the two other groups (Table I). This variation from the expected course occurred in all three experiments but was most decided among those animals that had been exposed to artificial light for the longest time.

The time relations of the reaction are more difficult to interpret. The mean values for the three experiments (Table II) show a comparatively close agreement despite the differences obtained in the first and third experiments and the very irregular development of lesions shown by all groups of animals in the second experiment (see Text-fig. 1).

The situation is clarified somewhat by comparing time intervals (in days) from the development of primary to metastatic lesions and from the occurrence of edema to metastatic orchitis as follows:

From Development of Primary Lesions.

	Control	Dark	Light
Exp. I.....................	28.4	30.3	23.4
" II.....................	29.7	33.8	31.9
" III.....................	21.5	34.0	34.9
Mean.....................	26.5	32.7	30.1

From Occurrence of Edema.

	Control	Dark	Light
Exp. I.....................	19.5	16.1	9.2
" II.....................	12.1	16.6	11.7
" III.....................	10.8	16.0	24.7
Mean.....................	14.1	16.2	15.2

This analysis shows that as we narrow down our basis of reckoning from the rather remote event of inoculation to the occurrence of the reaction immediately preceding the development of metastatic orchitis, relations that are more or less obscure become clearly defined. It is at once apparent that conditions that determined the time of development of metastatic orchitis were not the same in any two groups of animals. The light group and the controls are diametrically opposed whereas the animals in the dark maintain a constant relation from one experiment to another just as they did in the development of primary lesions and edema. In view of what has been said with respect to the significance of the time relations of the reaction, it is important to note that the animals exposed to artificial light showed a progressive prolongation of the reaction time.

Generalized Lesions.—The occurrence or non-occurrence of generalized lesions, the character and extent of the lesions, the time of their appearance, the duration of the period of active development, and the persistence of the lesions afford the most acceptable basis for estimating the effectiveness of the reaction to syphilitic infection in the rabbit. As is well known, the course of disease is subject to wide variation in all of these respects, but, as a rule, is comparatively constant under a given set of conditions.

With respect to the incidence of generalized syphilis, the figures given in Table I show that the condition was definitely more frequent among the animals in the dark and slightly less frequent among those in the light than among the controls. It will be noted, however, that there were fewer cases of generalized syphilis among the control animals of the second and third experiments than in the first and a marked reduction in the number of foci affected (focal distribution, Table I). The animals in the light also showed a reduction in the incidence and distribution of lesions which followed a slightly different order but those in the dark showed very little variation in either of these respects. The relations that obtained are shown graphically in Text-fig. 2.

The apparent reduction in the incidence and distribution figures for the dark group of animals in the second experiment is probably due to a change in the character of the lesions. In the first experiment, the majority of lesions in all three groups of animals were comparatively large and of a destructive character. This was especially

true of controls and of animals in the dark, and in both of these groups there were several cases of unusually severe generalized syphilis. Among the light animals, however, the lesions were smaller, less destructive, and of shorter duration.

There was no material change in the character of the lesions presented by animals of the control and light groups in the second and third experiments. Among the animals in the dark, however, the character of the disease changed. The first lesions tended to be small and of short duration, but were prone to relapse and some of the recurrent lesions attained a large size. This peculiarity of the reaction was at first confusing and, in the second experiment, it was not until some of the lesions had recurred a second or perhaps a third time that the significance of the condition was fully appreciated and a definite diagnosis of generalized syphilis was made. It is probable, therefore, that the incidence and distribution figures for these animals in the second experiment are too low and it is certain that the time given for the occurrence of the first generalized lesions is too long; hence, some allowance should be made for this error in the calculations which involve the time of occurrence of these lesions. The values given for the third experiment are little if at all affected by this factor.

The time relations of the reaction to generalized infection may be viewed in a number of ways. The figures given in Table II show no striking difference in the time from inoculation to the development of generalized lesions in the three groups of animals. The relations change from one experiment to another, but one is impressed chiefly by the closeness of the agreement and the narrow limits of variation. The mean results for the three experiments suggest a precocious development of lesions among the animals in the dark and perhaps a retarded development in the light group if we consider the time at which the first generalized lesions appeared, that is the onset of the generalized reaction, as shown by the following tabulation:

	Control	Dark	Light
Exp. I...................	54.9	53.9	58.8
" II...................	55.0	59.5	63.9
" III...................	63.7	53.1	56.2
Mean...................	57.9	55.5	59.6

If we take the mean time of development of all generalized lesions, the situation is slightly different. The results in this case are as follows:

	Control	Dark	Light
Exp. I......................	65.5	60.4	65.2
" II......................	69.4	67.1	63.1
" III......................	65.0	61.1	61.1
Mean.....................	66.6	62.9	63.1

Considered from this point of view there is a suggestion that generalized lesions developed earlier on the whole in both the dark and light groups than in the controls.

The results as to the duration of the period of active secondary eruption are more conclusive. The mean time of appearance of the last lesions in each animal was as follows:

	Control	Dark	Light
Exp. I......................	82.4	76.4	73.4
" II......................	70.3	84.1	66.4
" III......................	72.4	72.4	65.0
Mean.....................	75.0	77.6	68.3

This definitely indicates an earlier termination of the period of secondary reaction in the animals of the light group and a probable prolongation of the period in the dark group. Greater differences are shown, however, by a comparison of the mean time between the development of the first and the last lesions in all animals of the three groups as indicated below:

	Control	Dark	Light
Exp. I......................	27.5	22.5	14.6
" II......................	15.3	24.6	2.5
" III......................	8.7	19.3	8.8
Mean.....................	17.2	22.1	8.6

These figures show not only a difference in the character of the reaction, but they furnish an excellent index of the efficiency of the reaction developed by the three groups of animals.

There is one other comparison that may be made for the purpose of bringing out an important relation between the occurrence of generalized lesions and metastatic orchitis. The time interval between these reactions may be estimated from the appearance of the first generalized lesions or from the mean time for all lesions. The respective results are:

From Metastatic Orchitis to Time of First Generalized Lesions.

	Control	Dark	Light
Exp. I.....................	13.9	11.2	22.4
" II.....................	9.2	12.9	17.6
" III.....................	24.1	7.8	6.1
Mean.....................	15.7	10.6	15.4

From Metastatic Orchitis to Mean Time for All Generalized Lesions.

	Control	Dark	Light
Exp. I.....................	24.5	17.7	28.8
" II.....................	23.6	20.5	16.8
" III.....................	25.4	15.8	11.0
Mean.....................	24.5	18.0	18.9

The results obtained by these comparisons show that, while in successive experiments the time between the occurrence of metastatic orchitis and the initiation of the reaction to generalized infection in the control animals was irregular, the mean time between the two reactions was virtually constant. This was not true of either of the experimental groups. The initial time between the two reactions was affected in precisely the same manner and, while from one experiment to another the animals in the dark displayed less variation than those in the light, both showed a progressive reduction of the reaction time. That is, the longer the animals had been exposed to a given condition, the shorter the interval between the development of metastatic orchitis and generalized lesions.

These results are of interest on account of their bearing on the question of the efficiency of the reaction displayed. In the case of animals in the dark, the significance of the results is at once apparent. The short interval between the development of the two sets of lesions was associated with a high incidence of metastatic orchitis and generalized lesions and is to be regarded, therefore, as evidence of an ineffectual reaction. But, the occurrence of a similar reduction in the reaction time of the light animals was associated with two unusual conditions, namely, absence of infection in the uninoculated testicle of some animals and delayed development of lesions in others; in addition, the incidence of generalized lesions was low. It is evident, therefore, that the efficiency of the reaction displayed by these animals was of a higher order, in fact, sufficiently potent to disturb the usual course of events to such an extent as to cause a gradual overlapping of metastatic orchitis and generalized lesions (see Text-fig. 1) but not sufficient to entirely prevent the occurrence of either of these conditions. In the end, generalized lesions actually increased in proportion to the suppression of the reaction in the uninoculated testicle due undoubtedly to the failure of the protection usually afforded by such reactions, hence, the reaction time in the light animals bears an inverse relation to the suppression of lesions in the uninoculated testicle or to the inhibitory effect of the reaction in the inoculated testicle.

This result is not uncommon with strains of *pallidum* of low virulence but is very unusual with strains of high virulence, as under ordinary circumstances, the reaction in the inoculated testicle is rarely sufficient to prevent or materially delay the occurrence of metastatic orchitis. The infection progresses virtually unimpeded until lesions develop in both testicles and not until then does the reaction become sufficiently potent to prevent the occurrence of other lesions. It is evident, therefore, that the primary reaction in the light animals was unusually effective and the same may be said of the secondary reaction as the incidence of generalized lesions was comparatively low and the duration of the period of active development was unusually short.

Recovery.—A final comparison of the behavior of the three groups of animals may be made on the basis of the proportion of animals of each group that showed complete resolution and healing of all lesions

within the period of 4 months' observation. The results, expressed in per cent, were as follows:

	Control	Dark	Light
Exp. I	30.0	50.0	70.0
" II	10.0	50.0	40.0
" III	50.0	40.0	30.0
Mean	30.0	46.6	46.6

Here, again, we find that the results for the controls are irregular while those for the animals in the dark are fairly uniform and the light animals show a progressive reduction in the percentage of recoveries with mean values for both the light and dark groups that are higher than those for the controls. The peculiar feature of these results is the diminishing percentage of recoveries among the light animals which is attributable to a combination of circumstances analogous to those that were pointed out in the discussion of the relations between metastatic orchitis and generalized lesions; that is, an early partial suppression of the infection with consequent delay in the development of full immunity.

Some additional evidence as to the influence of the light environment may be adduced from the records of weight which give a fair index of the physical condition of the three groups of animals during the period covered by the experiments. These results may be tabulated in the form of percentage gains in weight from the beginning to the conclusion of the experiments.

	Control	Dark	Light
Exp. I	21.4	33.5	39.8
" II	38.1	25.6	53.9
" III	31.2	34.1	43.3
Mean	30.2	31.1	45.7

These results are of essentially the same order as those obtained for various phases of the reaction to infection. In fact, the resemblance is so close that one can hardly escape the conclusion that the two sets of results represent closely related effects of a common cause.

The interpretation of results will be facilitated by calling attention to one other feature of the experiments, namely, the differences in the severity of the disease shown by control animals of the three experiments in relation to prevailing conditions of sunlight. By reference to tables and text-figures, it will be seen that, in the first experiment, the infection was much more severe than in the second or third. The comparative results for incidence and distribution of generalized lesions were:

	Incidence	Actual distribution	Relative distribution
Exp. I.....................	80.0	8.0	6.4
" II.....................	70.0	4.1	2.9
" III.....................	70.0	4.1	2.9

The monthly hours of sunshine (Central Park Observatory, New York) for the period immediately preceding and covered by the experiments were:

	Mean normal	Actual	Departure from normal
August......................	272.3	316.0	+43.7
September...................	230.4	210.4	−20.0
October.....................	200.8	193.3	−7.5
November...................	152.6	184.0	+31.4
December...................	140.2	168.2	+28.0
January.....................	139.7	161.8	+22.1
February....................	158.5	164.3	+5.8
March......................	203.7	274.2	+70.5
April.......................	227.7	280.9	+53.2

The points of interest brought out by these figures are, first, the marked reduction in sunshine immediately preceding and following the inoculation of the first group of animals (Experiment I), second, the (unusually) high value for November, and, third, the maintenance of an almost constant and unusually high level of sunshine for the months of December, January, and February which covers the critical period of the second and third experiments. As will be seen, the actual level of sunshine for the 3 months was slightly higher than the normal November and February level or represented a mid-fall and early spring rather than a winter condition.

Without attempting to analyze these relations in detail, it may be pointed out that the occurrence of the most severe infection followed an abrupt and decided change in the prevailing level of sunshine and that, with the stabilization of the sunshine curve at a slightly lower level, the infection assumed a milder form; there were irregularities in the progress of the disease, just as the hours of sunshine varied from week to week, but the final results for the second and third experiments were essentially the same. These facts have a direct bearing on the results obtained in other groups of animals and are of especial interest in connection with effects produced by constant exposure to artificial light.

In attempting to arrive at a conclusion concerning the significance of the results reported, the distinctive features of the reaction displayed by each group of animals should be viewed in relation to the nature of the experimental condition represented. The variable factor in the experiments was the light environment. Other factors were either constant or the variations that occurred were common to the three groups of animals. For reasons that have been explained, the conditions compared were extreme; with one group of animals, light was excluded so that if it were capable of influencing the reaction to infection, this influence would be completely eliminated. The course of disease in these animals was characterized first by uniformity or constancy with respect to both the incidence and the time of occurrence of successive reactions or lesions and second, by a high incidence of generalized syphilis of a severe character. That is, the infection pursued a uniform course from inoculation to the development of outspoken secondary syphilis with comparatively little restraint during the early stages of infection.

A second group of animals was provided with a constant and continuous supply of artificial light of high intensity with a view to affording an opportunity for the development of any effect which the light might be capable of producing, free from any possible disturbing influence of periodic variation. In this case, the severity of the disease diminished with successive experiments as the length of exposure to light increased. There was an increased intensity of primary reactions with a progressive delay and diminished frequency in the occurrence of secondary reactions including metastatic orchitis and generalized

lesions. The picture presented suggested an accentuation of primary reactions with increased effectiveness and consequent delay or prevention of other lesions—a condition almost diametrically opposed to that presented by the animals from which all light was excluded.

It should be borne in mind, however, that placing the animals in darkened or artificially lighted rooms entailed a change in the light environment to which they were accustomed. This may have affected the results of the first experiment in particular and affords a possible explanation for the fact that, among the dark animals, the disease attained its maximum severity in the first experiment, was somewhat milder in the second, and again more severe in the third experiment.

The condition represented by the control animals differed from that of the animals receiving artificial light in three essential respects, namely, the character of the light, and the intensity and constancy of illumination. The results obtained differed also with respect to the general character of the reaction to infection, the relative frequency of occurrence of various reactive phenomena, and especially with respect to the regularity in the evolution of the disease from one experiment to another; but, the final results showed an evident tendency in the same general direction.

On the whole, it may be said that the results tended to conform to the nature of the experimental condition represented. In the control animals, the reaction to infection varied in accordance with the conditions under which the animals lived; the animals provided with artificial light showed a progressive increase in the efficiency of the reaction corresponding with the length of exposure to the light while among those in the dark there was a gradual lowering of the efficiency of the reaction to infection, if we measure efficiency in terms of prevention or delay in the development of successive manifestations of disease.

It is obvious that these effects are attributable to the conditions under which the animals lived rather than to any condition inherent in the material used for inoculation or to chance variation. It is also evident that the course of disease in the three groups of animals may have been influenced in a common direction by a number of unknown factors, including the material inoculated and there is unmistakable evidence of the action of such factors. The differences shown, how-

ever, can be accounted for only on the basis of differences in the light environment, the effect of which may have been accentuated or diminished by the influence of other factors. Moreover, it is apparent that the reaction of animals may be influenced by conditions to which they are exposed either before or after the initiation of the infection.

The mechanism concerned in the production of these effects is unknown, but since it has been shown that the light environment influences the physical constitution of normal rabbits (5) and that the organs most affected are organs that are concerned in the reaction to syphilitic infection (2, 8), it is logical to assume that the effects produced in this case represent expressions of functional activity which are closely related to changes in physical constitution.

Finally, the greater efficiency of the reaction displayed by animals exposed to artificial light as compared with those living in darkness and the maintenance of a better physical condition speak for a beneficial influence of light which is supported by the results obtained in control animals. There are, however, no grounds for assuming that the optimum effect was attained by the particular conditions that were employed in these experiments.

A comparison of effects due to filtered sunlight and to artificial light *per se* cannot be made as the condition complex represented by one form of light was variable and uncontrolled while that of the other was constant. The superiority shown by the artificial light may have been referable to the constancy of exposure rather than to differences in either the quality or the intensity of the light. It is evident that changing conditions of light environment are a disturbing factor and that in extreme cases the occurrence of such changes may completely counterbalance any beneficial effects of the light itself; but, the relative importance of the several factors that determine the effects of light on the animal organism and the general scope of their action are still uncertain.

SUMMARY.

A series of experiments was carried out for the purpose of determining whether the reaction of rabbits inoculated with *Treponema pallidum* might be influenced by their light environment. The condi-

tions compared were (1) diffuse sunlight filtered through window glass and subject to variations due to natural causes, (2) constant and continuous exposure to artificial light with a wave-length of from 3022 to 5790 Ångström units (Cooper Hewitt), and (3) complete exclusion of light.

The results showed clearly that each of these conditions produced a distinctive effect and that the effect tended to conform to the nature of the environmental condition. In general, the efficiency of the reaction to infection increased with the amount of light received and with the constancy of the exposure.

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AN INFECTIOUS GRANULAR VAGINITIS OF COWS.

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PLATE 15.

An infectious disease of cows characterized by an acute inflammation of the vaginal mucosa and terminating in the formation of raised red nodules studding the mucosa has been recognized in many parts of the world. Hess[1] reported that in certain sections of Switzerland over 60 per cent of the cows suffered with the disease. It is one of the common disorders of cows in this country.

Ostertag,[2] Hecker,[3] and others succeeded in cultivating a Gram-negative streptococcus from the mucopurulent exudate. The organism was described as extracellular and occurred in chains of from 6 to 9. In many instances it was associated with staphylococci and *B. coli*. Ostertag inoculated the vagina of cattle, sheep, goats, pigs, and mares with the streptococcus and reproduced the disease. He points out that Raebiger,[4] Jüterbock,[5] and others reproduced the disease in cows by intravaginal inoculation with a similar streptococcus. Blaha[6] observed in a series of cases bodies embedded in the epithelial cells similar in many respects to those observed in trachoma, which led him to believe that it was a Chlamydozoa infection.

Relatively little concerning the etiology of vaginal infections has been published in this country. Starr[7] noted that the nodules resulted from hyperplasia of the lymph follicles as the result of irritation. He succeeded in cultivating a streptococcus of the *viridans* type from the exudate.

[1] Hess, cited by Ostertag.[2]

[2] von Ostertag, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 269.

[3] Hecker, *Berl. tierärztl. Woch.*, 1900, 445.

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The disease we encountered resembled in many respects the usual granular vaginitis. However, it differed from the latter in that it was often more severe, and streptococci were not found in great numbers. It will be brought out later that the streptococci we isolated are not the microbic incitant of the condition.

History of the Cases.

A considerable proportion of our cases occurred during the months of November and December, 1925, and January and February, 1926. The disease became epidemic during November shortly after a tuberculin test when a large number of newly purchased cows and a considerable number of young native cows were introduced into the herd. In several instances all the newly purchased cows and the young native cows in certain barns were attacked about the same time. The epidemic subsided but sporadic cases continued to appear in native young cows introduced into the herd during the first 5 months of 1926. During this outbreak over 100 cases occurred.

In addition to this material we had access to several cases in cows originating in Ohio and purchased from a dealer, also to cases evidently originating in Oregon among cows shipped from there to New Jersey in special cars. This material convinces us that the infection with which we had to deal is one of considerable distribution in this country.

Characterization of the Disease.

The disease was severe among the newly purchased and young native cows. The vulva was greatly swollen and tender. The visible vaginal mucosa was deeply congested and swollen and the clitoris enlarged and bright red. The mucosa covering the floor and walls of the vagina was sprinkled with numerous, tiny, indistinct, grayish white areas which rapidly coalesced to form large plaques of grayish or yellowish white exudate (Fig. 1). When the exudate was forcibly removed a raw, bleeding, grayish red surface was exposed. Considerable thick mucopurulent exudate often gathered about the clitoris and on the floor of the vagina. The inflammation slowly subsided and the exudate sloughed exposing a granulating surface. The mucosa regenerated but tiny, round, red areas appeared embedded in

the mucous membrane. These enlarged and finally became round, raised red nodules 1 to 2 mm. in diameter. A little mucopurulent exudate frequently persisted about the clitoris for a considerable period.

The lesions in the cows originating in Ohio and Oregon were much less severe. In both groups the vaginal mucosa of a number of animals was sprinkled with the red granules similar to those observed in the severe cases. In other instances a more acute condition was observed, and here the vulva was swollen and tender. The vaginal mucosa was bright red and sprinkled with strings of loosely adherent, yellowish white, purulent exudate. At times small amounts of mucopurulent exudate accumulated on the floor of the vagina. With the subsidence of the acute inflammation the characteristic granules commenced to appear.

As far as we could determine the disease was confined to the vagina. The general health was not noticeably affected. The milk yield remained normal.

Bacteriological Findings.

We attempted to demonstrate the presence of organisms in films of the exudate from fresh cases by means of heat fixation and staining with methylene blue or Gram's method. By such procedures we were able to recognize a relatively few organisms, usually streptococci or micrococci, but in insufficient numbers to account for the lesions. When rapidly dried films were fixed for 3 to 5 minutes in methyl alcohol and then stained for 30 to 40 minutes with a solution consisting of Giemsa's stain 2.0 cc., methyl alcohol 1.5 cc., distilled water 20 cc., or stained with carbolfuchsin diluted 1:20 in distilled water for 1 to 2 hours, we were able to demonstrate a considerable number of tiny delicate rods with well developed polar granules (Fig. 2). In many instances the cytoplasm between the granules stained feebly or not at all, so that the organisms resembled tiny diplococci, shown in Fig. 3.

The exudate is composed largely of leucocytes, epithelial cells, a few endothelial phagocytes, and considerable mucus. It was possible by obtaining portions of the exudate on sterile swabs and bringing the material to the laboratory to cultivate on the ordinary media

certain well defined types of organisms, such as streptococci, staphylococci, *B. coli*, and long, slender, Gram-positive rods, but in no instance was an organism encountered which resembled the bacillus met with in the films. After a considerable number of failures we were successful in obtaining it in pure culture. The procedure finally adopted was to transfer the exudate directly from the cow into the condensation water of a blood agar slant. Agar slants were prepared from veal infusion, and when slanted and cooled, 0.5 cc. of defibrinated horse blood was added. From the first tube, three others were inoculated in series, care being taken to flame the loop between each tube. The tubes were then sealed with sealing wax and incubated for 5 days at 38°C. As a rule Tube 1 contained streptococci and other types of organisms. Tube 2 contained in addition to streptococci clumps of tiny coccoids and a few tiny bacilli with well defined polar granules. Tube 3 was often to outward appearances sterile, or showed an indistinct haze in a narrow zone about the level of the condensation liquid between the tube wall and the agar; examination of the stained films, however, revealed small numbers of tiny coccoids in clumps and occasional well defined rods (Fig. 4). Tube 4 contained a pure culture or remained sterile. Transfers from the tubes containing only the coccoids and rods are usually successful, but the organism is pretty apt to grow only in the condensation fluid or between the agar and glass for four or five generations; after this time delicate flattened colonies with slightly raised centers appear on the slant.

The organism stains poorly after heat fixation, but films fixed in methyl alcohol stain well with Giemsa. It is Gram-negative and non-motile. The morphology varies. A constant finding in cultures is the densely packed masses of tiny coccoids (Figs. 4 and 6) or tiny rods (Figs. 4, 5, 6) containing polar granules. Free forms more or less elongated with well defined granules are likewise present. In later cultures (Fig. 5), the bacilli are larger and stain more deeply. All cultures passed through a phase in which growth was apparently going on more rapidly, but a final adaptation to the medium had not been reached. Here large clumps of the coccoids are plentiful, as well as extremely long filamentous forms containing large masses of deeply stained protoplasm and the tiny granules (Fig. 6). This

phase passes and finally there is a reversion to the clumps of coccoids and the tiny rods with polar granules. Cultures in blood broth reveal the general variations as illustrated in Fig. 7. It is to be observed that considerable variation in size exists. In the films of exudate the bacilli measure from 1 to 2μ in length. Bacilli of this length are common in all the cultures. The coccoids are exceedingly small, $1/3\mu$, but the probabilities are that they comprise the polar granules of bacilli whose central zones and cell walls fail to stain. The filamentous forms referred to vary from 10 to 45μ in length. Many show a tendency to fragment near the ends. Others may stain irregularly throughout their entire length.

Once a culture is established on blood agar it is readily transferred to blood broth. Thus far it has not been possible to establish growth in coagulated horse serum to which sterile calf serum water has been added or in serum agar. It will grow, however, in the condensation water of plain agar or ascitic fluid containing fresh tissue such as guinea pig spleen or kidney.

In blood broth to which 1 per cent of dextrose, lactose, saccharose, maltose, or mannitol was added, no fermentation was observed after 10 days incubation. Milk heavily inoculated with blood broth culture remained unchanged. Indole was not produced in sugar-free broth containing blood.

It seemed possible from the size of the coccoids that the organism might readily pass through the coarser Berkefeld filters. On four occasions we attempted filtration through candles V and N but the filtrates remained sterile. Inoculations from the filtrates after suitable incubation were also negative.

Pathogenicity of the Bacillus.

Rabbits weighing 2000 gm. withstand 2 cc. of blood broth culture injected intravenously. Guinea pigs of 300 gm. remain well when injected intraperitoneal with 0.5 cc. of culture. 1 cc. may cause death or produce a febrile reaction lasting several days. The injection of 2 cc. has always resulted fatally. Death results from peritonitis in 24 hours. The bacilli are found in the exudate in enormous numbers and can be cultivated from the heart's blood.

Heifer calves, 3 or 4 months old, and 2 year old heifers were in-

oculated into the vagina with culture. In every instance acute inflammation resulted. Granules similar to those observed in the spontaneous disease were always observed after the acute inflammation had subsided. The following experiment affords an example.

The mucosa of the vagina of unbred Heifer 1116 was brushed with a swab immersed in the condensation water of a 3 day blood agar culture of the bacillus in the third culture generation. There was no reaction during the first 24 hours. On the 2nd day the vulva was swollen and tender. The vaginal mucosa was bright red and swollen. Strings of yellowish white, purulent exudate adhered to the mucosa covering the floor and sides. On the 3rd day the swelling was more marked and there was considerable tenderness on manipulation. The whole mucosa was bright red and bled when brushed lightly with a sterile swab. Blood agar inoculated with exudate on this day resulted in pure cultures. The films of the exudate (Fig. 8) showed necrotic epithelial cells, leucocytes, mucus, and a moderate number of the characteristic bacilli. On the 4th, 5th, and 6th days the congestion and swelling were pronounced, and considerable exudate was present about the clitoris and adhered to the walls. Cultures made on the 5th day contained the bacilli. On the 9th day there was more exudate and the whole mucosa appeared to be granulating. After 11 days the mucosa was studded with barely visible, indistinct, grayish white areas. These were a little larger and more red in color on the 12th day, and on the 13th day were recognizable as distinctly visible, raised, red nodules. The nodules increased in size and finally on the 19th day appeared round, sharply raised, firm, 1 to 1.5 mm. in diameter. Cultures made on this day contained the bacilli.

The heifer was slaughtered 89 days after the inoculation. The granules were still visible in the mucosa of the vestibule and walls of the vagina. They did not extend into the uterus. Examination of material fixed in Zenker's fluid and stained with methylene blue revealed that the lymph follicles in the submucosa were hyperplastic. Some follicles were discrete, others were joined by bands of round cells. Over the smaller, more discrete, round celled accumulations the epithelium was normal, but that overlying the larger follicles was heavily invaded with round cells (Fig. 9).

Mention has been made that many of the cultures from the spontaneous cases contained streptococci. These were all of the non-hemolytic or green-producing type and resembled those described by F. S. Jones[8] as the type usually found in the vagina of healthy cows. We inoculated four heifer calves with the cultures. No inflammation resulted, but on subsequent inoculation with pure cultures of

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[8] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 735.

the rods from vaginitis acute inflammation resulted followed by the formation of typical granules. In these experiments the granules appeared from 5 to 7 days after infection.

We were unable to obtain material for histological study from acute spontaneous cases. On several occasions old cows leaving the herd for various reasons were inoculated into the vagina with material from severe cases, but the animals failed to contract the disease. They had probably passed through an attack of the disease and were resistant. Calves as a rule respond moderately only to inoculation with infectious material. In certain instances such inoculations induced severer inflammation and afforded some insight into the nature of the acute process.

The mucosa of the vagina of Calf 1240, 4 weeks old, was brushed with a swab containing exudate from two spontaneous cases. The usual type of acute inflammation followed. 4 days later the calf was slaughtered. At antemortem examination the mucosa was scarlet and sprinkled with strings of tenacious, yellowish white exudate. Evidently the method of slaughter, similar to that used in abattoirs, caused a blanching of the vaginal mucosa, since it was of a pale yellowish pink color along the floor and walls. Anterior to the clitoris was a red area situated within the mucosa. Other portions of the mucosa contained a few tiny, sunken, irregular, red patches. A little mucopurulent exudate was present on the mucosa of the floor. Inoculation of blood agar with this material developed cultures of the characteristic bacilli. The uterus was normal.

Histological examination of fixed and stained material revealed well defined necrosis of portions of the epithelium. In portions most of it had apparently sloughed so that the surface was covered with a thin layer of necrotic epithelial cells, degenerated leucocytes, and a little fibrin. The submucosa was edematous and infiltrated with leucocytes and round cells. The blood vessels were moderately engorged with red cells and contained excessive numbers of leucocytes and round cells. Other portions of the epithelium were intact. Lesions were not found in sections of the uterus.

From clinical examination of cases, exudate from such cases, and the histological material, we feel that the process may in part be pieced together. The bacilli attack the mucosa in certain foci. Here necrosis of the epithelium results. A little fibrin may exude beneath the epithelium. Leucocytes in large numbers invade the mucosa. The submucosa is invaded by round cells and leucocytes. The exudate and mucosa slough, followed by regeneration accompanied by

large accumulations of round cells in follicle-like masses in the submucosa. The large amount of exudate in the outbreak may be explained by a heavy infection with the bacilli so that the necrotic areas occurred close together and gave the appearance of a continuous membrane.

<div style="text-align:center">DISCUSSION.</div>

It is apparent that in the large outbreak we had to deal with a severe type of inflammation of the vagina. In certain respects the type of disease differed from that usually considered typical of granular vaginitis and that encountered in our cases drawn from other sources. From each group, however, we succeeded in isolating a similar organism. The acute inflammation in all cases terminated in the appearance of the characteristic granules in the submucosa.

In the outbreak several factors contributed to exalt the severity of the infection. The disease could easily be spread by thermometers during a tuberculin test. There had been a large number of young cows recently introduced into the herd and these animals with young native cows represented a large number of susceptible individuals. The method of spread was apparently direct from cow to cow, since in this herd animals are brushed and curried before milking. It appeared that all cows on one side of a barn were infected at about the same time. A favorable opportunity was thus created for the rapid spread of the inciting organism to a large number of relatively highly susceptible cows.

With the culture isolated from the severe cases it was not possible to produce the severe type of disease. We simply reproduced a condition similar to that found in the cows from Oregon and Ohio. It must be remembered, however, that we used for the purposes of inoculation relatively small doses of a feebly growing culture. The experimental disease was always well pronounced, accompanied by a mucopurulent exudate, and the acute process terminated in the formation of the characteristic granules. In sharp contrast are the entirely negative results after inoculation with non-hemolytic streptococci also isolated from the vaginal exudate.

Although the question of immunity produced by an attack is not

definitely proved, yet considerable resistance seems to result. We have noted on several occasions that old cows standing between severe cases did not contract the disease although infectious material must have been frequently brushed into the vagina. In two instances cows which had been exposed during the outbreak were inoculated intravaginal with material from severe cases and failed to develop the disease.

The bacillus isolated is apparently one not described before. That its distribution is widespread is indicated by its presence in cows from Oregon, Ohio, and New Jersey. Morphologically it resembles in certain respects the bipolar group in having polar granules and giving rise to the long involution forms during certain phases. However, it differs in many respects from usual organisms of this type. It fails to ferment dextrose, or any of the carbohydrates, produces no indole, and possesses relatively no pathogenicity for rabbits. It grows only in media containing blood or bits of tissue, and then only in the parts of the tubes containing little free oxygen. It is not an anaerobe. Thus far it has failed to grow in the unsealed tube. It possesses no hemolytic or proteolytic properties. At present its identification rests largely on morphological criteria, the difficulty with which it stains, and the inability to grow in media which do not contain fresh blood or tissue.

SUMMARY.

A disease of cows characterized by swelling of the vulva, acute inflammation of the vaginal mucosa, accompanied by a more or less profuse mucopurulent exudate is described. After the acute inflammation subsides the mucosa becomes studded with tiny, round, raised, red nodules which persist 2 or 3 months or longer. The acute lesion consists in necrosis of the epithelium and accumulations of leucocytes and round cells in the edematous submucosa. The nodules in the later stages are made up of densely packed masses of lymphocytes in the submucosa which force the epithelial layer outward.

A Gram-negative bacillus with tiny polar granules was found in the exudate. It measures 1 to 2μ in length and stains with difficulty. The organism was obtained in pure culture by inoculating the exudate

into tubes of slanted agar to which defibrinated horse blood had been added. Growth occurs only in sealed tubes. The organism possesses slight pathogenicity for guinea pigs. When freshly isolated cultures were introduced into the vagina of heifers or young calves, acute inflammation resulted which terminated in the characteristic granular stage of the disease.

EXPLANATION OF PLATE 15.

FIG. 1. Natural infection. Severe inflammation of the vagina. Note patches of exudate on the mucosa. About 1/5 natural size.

FIG. 2. The bacilli along the border of heavily stained mass of exudate. Spontaneous case. Methyl alcohol fixation. Dilute carbolfuchsin stain. × 1000.

FIG. 3. Exudate from spontaneous case, showing two large leucocytes and two bacilli with polar granules. Methyl alcohol fixation. Giemsa stain. × 1000.

FIG. 4. The condensation fluid from an original blood agar culture 5 days old. Note the clump of coccoids, a single well stained bacillus, and an elongated form. A red blood cell is also present. Giemsa stain, after methyl alcohol fixation. × 1000.

FIG. 5. The same culture as Fig. 4, in the fourth generation. Condensation fluid of a 5 day blood agar culture. The bacilli are larger and stain more intensely. A red blood cell is included in the field. Giemsa stain. × 1000.

FIG. 6. The same culture as Figs. 4 and 5, in the fourteenth culture generation. Condensation fluid of a 3 day blood agar culture. Three forms are illustrated, a large clump of short coccoids, a few individual bipolar forms, and two long filaments, one of which shows a tendency to fragment. Giemsa stain. × 1000.

FIG. 7. A blood broth culture, in the third generation, 3 days old. Giemsa stain. × 1000.

FIG. 8. The bacilli in the vaginal exudate from Heifer 1116, 3 days after intravaginal inoculation with culture. Giemsa stain. × 1000.

FIG. 9. Section of the mucosa of the vagina of Heifer 1116, 89 days after inoculation with culture. Note the infiltration of round cells in the mucosa and the dense accumulation of round cells in the submucosa. Zenker's fixation. Eosin-methylene blue stain. × 66.

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(Jones and Little: Infectious vaginitis of cows.)

EPIDEMIOLOGICAL STUDIES ON RESPIRATORY INFEC-
TIONS OF THE RABBIT.

IX. THE SPREAD OF BACTERIUM LEPISEPTICUM INFECTION AT
A RABBIT FARM IN NEW CITY, N. Y.

AN EPIDEMIOLOGICAL STUDY.

BY LESLIE T. WEBSTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

A comprehensive experimental study of the mode of spread of a
bacterial disease demands knowledge of its natural epidemiology
(1, *a*). This includes data describing the natural prevalence and
distribution of the inciting organism, the frequency of various bac-
terial types in nature, their normal portal of entry into the body of
the host, the different responses of the host to the microbe, and the
frequency of each response under varying hereditary and environ-
mental conditions. Such information regarding the natural
prevalence of an animal disease has at least two important uses in
experimental epidemiology,—first, it enables one to induce the in-
fection experimentally, under conditions which simulate nature
closely, thereby reducing the number of disturbing artifacts; second,
it serves as a control with which to evaluate the conclusions derived
from the precise, yet necessarily limited experiments of the laboratory.

As an aid to our experimental studies of *Bact. lepisepticum* infec-
tion of rabbits, we have observed the native disease as it occurs
among the stock at The Rockefeller Institute and at a rabbit farm at
New City, N. Y. The findings in the former group, during the years
1923 and 1924, may be reviewed briefly as follows.

Bact. lepisepticum infection has been found present in about 60 per cent of the
rabbits at The Rockefeller Institute (1, *b*). From 20 to 40 per cent of the unused,
"normal" stock showed clinical or pathological evidence of snuffles, 5 per cent

evidence of otitis media (1, *c*), 30 per cent were "healthy," nasal carriers (1, *d*), and 20 per cent appeared normal and were not carriers. Among the animals subjected to operation, or otherwise used for experimental purposes, the percentage of infection was greater—in one such series, 40 per cent showed evidence of snuffles as compared to 20 per cent in the corresponding control group (1, *c*); in another, otitis media, 45 per cent, as compared to 5 per cent in unused stock (1, *c*). Inoculations of injurious substances were found to increase the incidence of the "spontaneous" infection by as much as 50 per cent (1, *c*). Both "D" and mucoid types of *Bact. lepisepticum* were encountered at this time (1, *f*).

The New City group of rabbits was studied in a somewhat similar manner and the findings are described in the present paper.*

Source of Material.

The present studies were made at a commercial rabbitry in New City, N. Y.[1] The farm is situated in open country, 200 feet above sea level, in a sparsely populated community. The buildings are placed on a tract of land comprising several acres and are isolated by means of a surrounding wire fence, 8 feet high. A number of watch dogs serve as further protection from animal marauders. Three buildings were in use during the first months of our observations; later, a fourth was added. In general architecture, they are all similar (Text-fig. 1). They measure 100 feet by 20 feet, with the long dimensions facing east and west. Each building contains two rows of wooden cages, running the length of the structure, arranged in tiers of four from ceiling to floor. About 368 cages, measuring 5 feet by 2.1 feet by 1.6 feet each, are contained in each building. The cages consist of a front runway facing toward the open east or west exposure, and a rear partition, kept relatively dark and used for breeding. Each house is cared for by two men who maintain a uniform and efficient system of cage cleaning and feeding throughout. Temperature fluctuates during the year according to outside weather conditions. No artificial heat is used. The side doors, east and west, are thrown open whenever possible, thus making conditions practically "out-of-doors." Food consists of hay, a grain mixture of oats, oatmeal, and wheat, fresh carrots, cabbage, and greens, according to the season. During the winter cold storage carrots are used.

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* Since submitting the manuscript for publication data through October have been obtained and the text-figures and text have been changed accordingly.

The rabbit population is composed almost entirely of pedigreed and registered stock. About 50 per cent are Chinchillas, 15 per cent Blue Beverens, 10 per cent Havanas, 10 per cent Belgian hare crosses with Chinchillas, and 15 per cent Gaudas, Lilacs, and Angoras. Pure line breeds were thus available for comparative study.

The animals are distributed as follows: Building 2, all breeds; No. 3, Chinchillas; No. 4, Chinchillas, Blue Beverens, and Angoras; No. 6, Chinchillas. Each rabbit 6 months old or over occupies a separate cage. The females are bred four or five times a year; four males are used for twenty females. Complete breeding records are kept. The young remain in the cage with the mother until they are old enough to be placed in individual cages.

TEXT-FIG. 1. Building 6, showing open side doors and arrangement of individual rabbit pens. Photograph by Edwin Levick furnished by Mr. Haack.

Methods of Study.

The spread of *Bact. lepisepticum* infection among this group of rabbits was studied as follows. Animals 6 months of age or over, occupying individual cages, were selected for observation. The number of these varied from 841 to 1325. The young were disregarded.

(1) From November, 1924, until November, 1926, complete mortality records were kept. (2) From November, 1924, until April, 1925, every rabbit that died was wrapped in paper, placed on ice, and sent to The Rockefeller Institute, where a complete autopsy was performed and cultures were taken. (3) During November, 1924, February, May, and August, 1925, a clinical examination of each rabbit was made. Its general condition was noted, signs of *Bact. lepisepticum* infection were searched for carefully, and other abnormalities were recorded. (4) Finally, a group of clinically normal Chinchillas and Blue Beverens was tested for fre-

TABLE I.

Summary of Data on the Mortality and Incidence of Snuffles among the Rabbits at the New City Farm, N. Y.

Date of observation	Total No. of rabbits examined	Total per cent snuffles	Total mortality per 1000	Bact. lepisepticum infection mortality per 1000	Intestinal infection mortality per 1000	Miscellaneous causes mortality per 1000	Chinchilla breed — Total No.	Chinchilla Mortality No.	Chinchilla Mortality Per cent	Chinchilla Snuffles No.	Chinchilla Snuffles Per cent	Havana breed — Total No.	Havana Mortality No.	Havana Mortality Per cent	Havana Snuffles No.	Havana Snuffles Per cent	Blue Beveren breed — Total No.	Blue Beveren Mortality No.	Blue Beveren Mortality Per cent	Blue Beveren Snuffles No.	Blue Beveren Snuffles Per cent
1924																					
November	1016	46.2	30.5	14.75	8.86	6.9	576	19	3.3	285	49.4	117	5	4.2	50	42.7	82	1	1.2	20	24.3
December	—	—	*48.3	23.6	16.75	7.9	—	27	4.7	—	—	—	8	6.8	—	—	—	4	4.8	—	—
1925																					
January	—	—	*40.3	24.6	12.8	2.95	—	31	5.3	—	—	—	9	7.7	—	—	—	1	1.2	—	—
February	1015	48.9	27.6	12.8	10.9	3.94	503	19	3.8	268	53.2	118	4	3.4	64	54.2	112	1	0.9	39	34.8
March	—	—	*47.4	27.6	11.8	7.9	—	26	5.1	—	—	—	7	5.9	—	—	—	2	1.8	—	—
April	—	—	*75.9	—	—	—	—	40	7.9	—	—	—	15	12.7	—	—	—	3	2.6	—	—
May	1116	49.1	106.7	—	—	—	528	64	12.1	287	54.3	118	18	15.3	68	57.6	196	11	5.6	54	27.5
June	—	—	*89.5	—	—	—	—	57	10.8	—	—	—	11	9.3	—	—	—	4	2.0	—	—
July	—	—	*28.7	—	—	—	—	20	3.8	—	—	—	4	3.4	—	—	—	1	0.5	—	—
August	1308	43.3	28.1	—	—	—	564	23	4.0	284	50.3	117	6	5.2	78	66.6	214	1	0.3	68	24.1
September	1325	—	*31.2	—	—	—	630	17	3.0	—	—	—	4	3.4	—	—	325	8	2.5	—	—
October	1310	—	28.7	—	—	—	620	17	2.7	—	—	—	4	3.4	—	—	350	5	1.4	—	—
November	—	—	24.4	—	—	—	—	10	1.6	—	—	—	—	—	—	—	350	6	1.7	—	—
December	1113	—	39.5	—	—	—	550	18	3.3	—	—	—	—	—	—	—	300	14	4.7	—	—
1926																					
January	1087	—	37.8	—	—	—	550	22	4.0	—	—	—	—	—	—	—	285	9	3.20	—	—
February	971	—	36.0	—	—	—	490	16	3.3	—	—	—	—	—	—	—	260	5	1.9	—	—

Comparative snuffles and mortality rates in different breeds of rabbits

March...........	954	—	24.0	—	—	—	490	14	2.9	—	—			230	4	1.7	—	—
April...........	1016	—	33.5	—	—	—	490	22	4.5	—	—			250	6	2.4	—	—
May...........	961	—	34.0	—	—	—	441	26	5.9	—	—			240	4	1.7	—	—
June...........	981	—	26.0	—	—	—	458	19	4.1	—	—			237	6	2.5	—	—
July...........	868	—	50.0	—	—	—	430	22	5.1	—	—			206	13	6.3	—	—
August...........	841	—	19.0	—	—	—	470	7	1.5	—	—			185	3	1.6	—	—
September...........	893	—	25.0	—	—	—	489	13	2.7	—	—			220	0	0	—	—
October...........	855	—	14.0	—	—	—	450	6	1.3	—	—			222	0	0	—	—

* Rates based on total population of last recorded census.

quency of nasal carriers of *Bact. lepisepticum*. Thus, we have available for comparative study mortality, morbidity, and carrier records on several pure line strains of rabbits over a considerable period of time.

RESULTS.

The results of these observations are summarized in Tables I to III, and are described under three main heads,—those pertaining to (1) the entire rabbit population; (2) to various breeds; and (3) to individual animals. We have included only such data as seemed significant; a mass of incidental information collected for the sake of completeness will be referred to briefly.
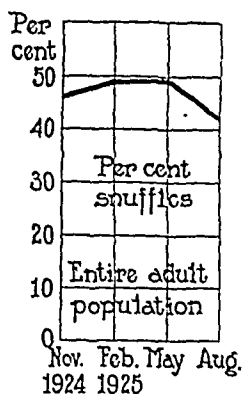
Text-Fig. 2. Incidence of clinical snuffles among the adult rabbits at New City farm, N. Y.

Bacteriology.

At the outset, it may be stated that with very few exceptions *Bact. lepisepticum* was obtained from the cases of pneumonia and septicemia examined. It was always recovered from the nasal passages of animals showing clinical evidence of snuffles. Furthermore, a considerable percentage of apparently healthy animals proved to be nasal carriers of this organism.

In every instance the bacterial type obtained was mucoid, with cultural and serological characteristics similar to mucoid strains obtained from rabbits at The Rockefeller Institute and from a rabbit colony at Ray Brook, N. Y. (2). The virulence was uniform and constant; instilled intranasally into young animals, pneumonia was

infrequently induced, snuffles in 40 to 50 per cent of the animals, and "healthy" nasal carriers in 20 to 30 per cent. Detailed studies of some of the strains are described elsewhere (1, f).

Detail of wall

| Doors | Window | Doors | Window | Doors |
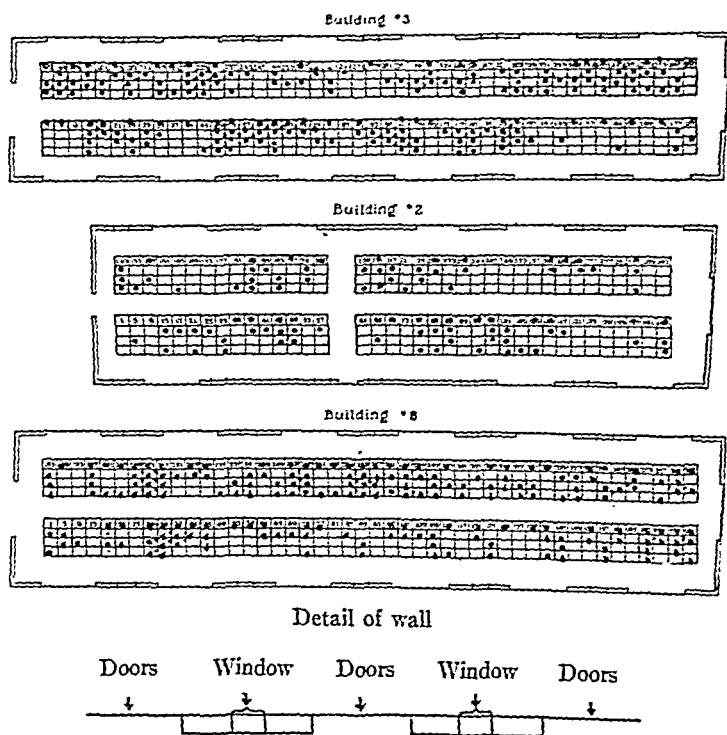
TEXT-FIG. 3.

TEXT-FIGS. 3 to 6. Distribution of clinical snuffles among the adult rabbits at New City farm, N. Y., November, 1924 (Text-fig. 3), February, 1925 (Text-fig. 4), May, 1925 (Text-fig. 5), and August, 1925 (Text-fig. 6).

Findings Referable to the Total Adult Population.

The incidence of clinical snuffles throughout the total adult population was determined in November, 1924, and in February, May, and August, 1925 (Table I). Cases were found to differ in severity and to vary in this respect from month to month. However, all showed the white, purulent, nasal discharge, matted paws, heavy breathing, and sneezing characteristic of *Bact. lepisepticum* infection. Indeed,
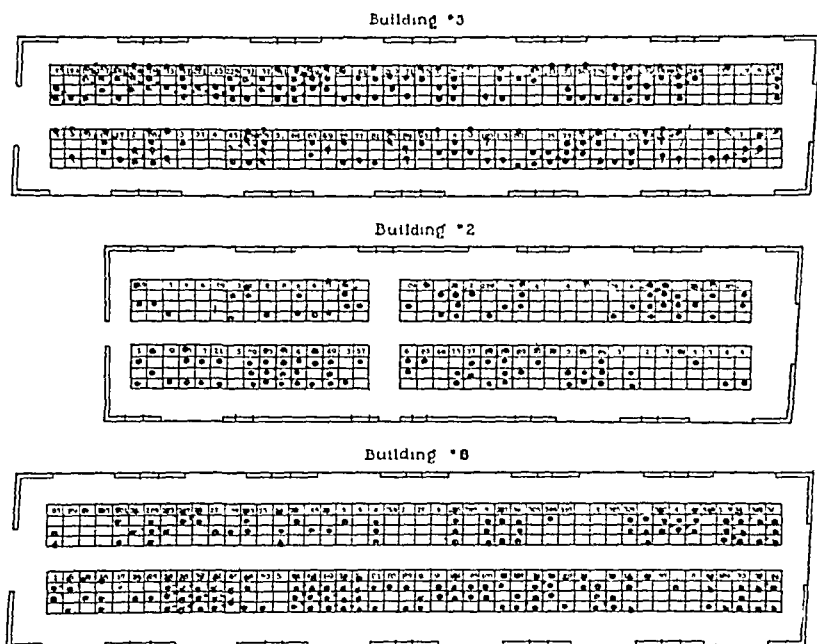
TEXT-FIG. 4. Clinical snuffles, February, 1925.
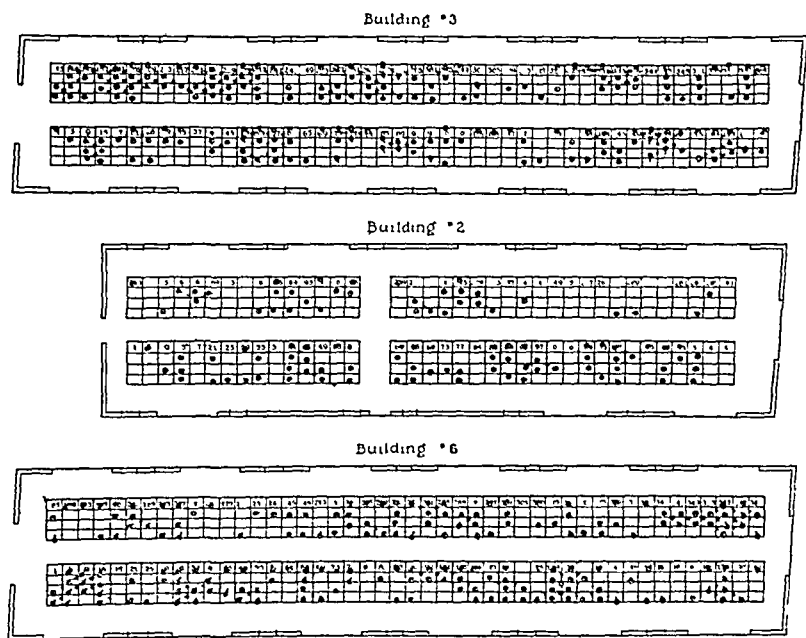
TEXT-FIG. 5. Clinical snuffles, May, 1925.

whenever cultures were taken from the nasal passages, *Bact. lepi-septicum* of the mucoid type (1, *f*) was obtained in great numbers.

The results of these examinations are plotted in Text-fig. 2. The rate per hundred of clinical snuffles rose from 46 in November to 49 in February and May, and dropped to 42 in August. Thus a definite spring rise was noticeable, similar to that observed among the rabbits at The Rockefeller Institute. Among this latter group the average percentage of snuffles varied between about 20 and 60 per cent and exhibited definite spring and fall rises (1, *b*).
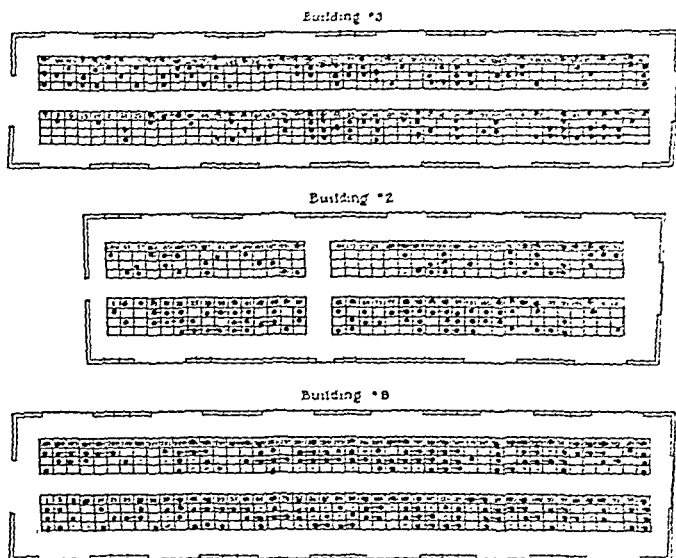
TEXT-FIG. 6. Clinical snuffles, August, 1925.

The distribution of the snuffles cases is charted in Text-figs. 3 to 6. In Text-fig. 3, the spot map for November, 1924, the infection is shown to be present generally throughout the buildings but tending to a focal distribution within each. Thus, in Building 3, definite areas of snuffles are indicated from Cages 13 to 33, 49 to 77, 89 to 109, and 117 to 137. On the opposite tier, there appear to be more cases at either end than in the middle. In Building 2, the infection was less frequent and more scattered. Text-figs. 4 to 6,

which indicate the February, May, and August distribution, show the various foci spreading locally from cage to cage, or disappearing in the same manner.

These maps, together with the mortality records, were useful in demonstrating two facts; first, that *Bact. lepisepticum* infection in the various buildings was widespread and relatively similar in amount, and second, that the location of doors and windows bore no special relation to the extent or distribution of the disease.

Specific mortality rates from November, 1924, to April, 1925, were obtained by autopsying each rabbit dying within this period and
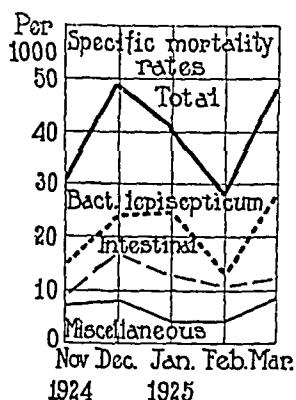
TEXT-FIG. 7. Specific mortality rates among the adult rabbits at New City farm, N. Y.

determining as far as possible the cause of its death (Table I). *Bact. lepisepticum* pneumonia and septicemia proved responsible for more than 50 per cent of the total mortality, intestinal disturbances of undetermined etiology[2] for about 30 per cent, and miscellaneous causes for less than 20 per cent (Text-fig. 7).

Text-fig. 7 shows that the fluctuations in deaths due to *Bact. lepisepticum* coincided with the variations in total mortality. During March, 1925, modifications in the green food diet were instituted and thereafter it became apparent, although no autopsies were performed,

[2] No recognized pathogenic organism was recovered from these cases. Occasionally, pin-worms were present, but not in sufficient numbers to be considered the cause of death. Distention of the ileum, cecum, and colon, with occasional subserous petechiæ and flecks of fibrin were the only pathological findings at autopsy.

that deaths from intestinal disturbances were reduced and that at least 75 per cent of the total mortality was the result of *Bact. lepisepticum* infection. Hence, it will be assumed that the trend of the total mortality curve throughout the 2 year period of observation is a general index of mortality fluctuations due to *Bact. lepisepticum.*

The rate of total mortality per month from November, 1924, to November, 1926, is shown in Text-figs. 8 and 9. During both the 1924–25 period (Text-fig. 8) and the 1925–26 period (Text-fig. 9),
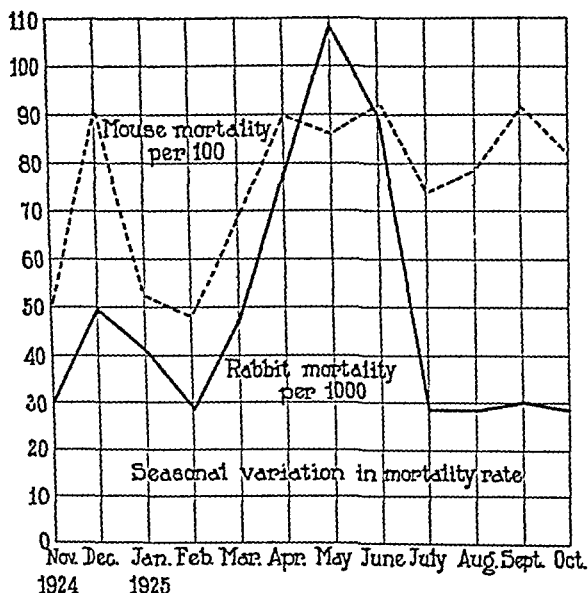
Text-Fig. 8. Fluctuations in total mortality of adult rabbits at New City farm, N. Y., 1924–25.

fluctuations occurred in well defined waves, being highest in the spring, low in summer, with a rise again in the fall.

A second curve is included on each chart, representing monthly variations in mortality of mice following the administration of a fixed dose of mouse typhoid bacilli.

The experiments with mice were carried out in such a way as to eliminate as far as possible all disturbing variables (1, *a*). On about the 15th of each month, 50 mice of the inbred Rockefeller Institute strain, 12 to 14 weeks old, weighing

18 to 20 gm. each, bred and raised under the same environmental conditions, given the same diet since birth, and free of any previous exposure to these organisms, were given *per os*, by stomach tube, a fixed dose of about 4,000,000 mouse typhoid bacilli from a standard fluid culture obtained from an agar slant kept at 4°C. (3). Because of the control and elimination of variables in these experiments and because the mortality of a number of groups of similar mice inoculated at the same time differs by a daily average of less than 5 per cent, we consider the wave-like fluctuations in mouse typhoid mortality to be due to variables associated with the season of year.
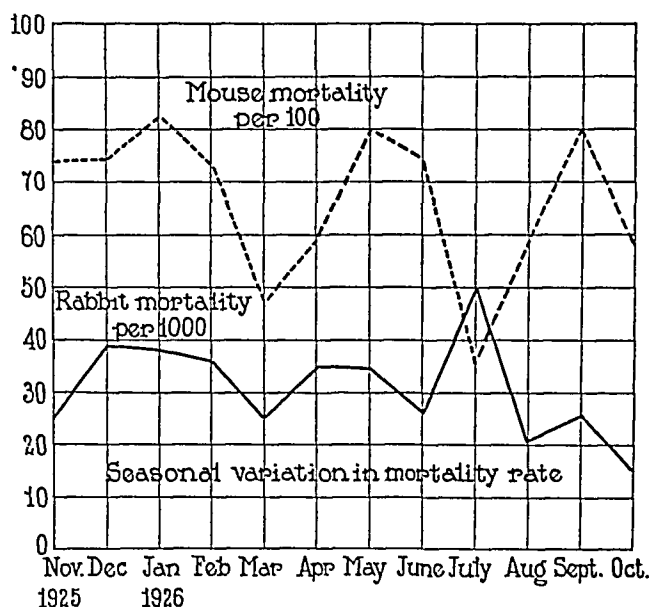
Text-Fig. 9. Fluctuations in total mortality of adult rabbits at New City farm, N. Y., 1925–26. From November, 1925, to April, 1926, the mouse curve appears as a fine dotted line. This has been done to indicate a slight change in the technique of the mouse experiments during this period, which changed the absolute values but not the trend.

For 2 years the fluctuations of the mouse typhoid and rabbit mortality curves have shown a remarkable degree of similarity. During 1924–25 (Text-fig. 8) the peaks in December, May, and September, and low points in February and July coincided, while in 1925–26 (Text-fig. 9) the peaks in January, May, and September, and the depressions in November and March were again quite similar. We regard the mouse typhoid curves as quantitative measurements

of the influence of seasonal variables on mortality from mouse typhoid under the conditions of this experiment, and in calling attention to the striking similarity of these curves to the total rabbit mortality in the community at New City,[3] we suggest the possibility that the latter, although relatively uncontrolled, are nevertheless to be interpreted as expressions of variations in host resistance associated with the seasons.

These several observations relative to the total rabbit population may be summarized briefly as follows. The incidence of clinical
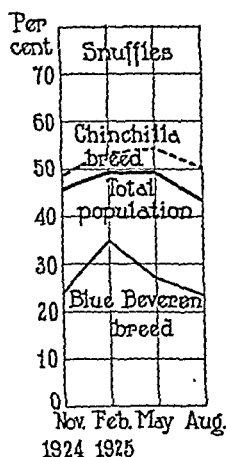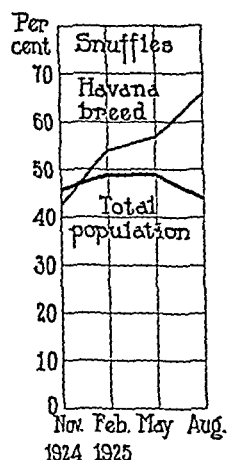
TEXT-FIG. 10.   TEXT-FIG. 11.

TEXT-FIG. 10. Comparative incidence of snuffles of Chinchilla and Blue Beveren breeds.

TEXT-FIG. 11. Incidence of snuffles among the Havana breed.

snuffles in the group of about 1000 adult rabbits varied from 46 to 49 to 42 per cent during a period of 10 months, and showed a definite spring rise. The total mortality among these animals, 50 to 75 per cent of which proved to be due to *Bact. lepisepticum* infection, showed wave-like fluctuations over a period of 2 years. High peaks occurred in the spring and fall, and low points were reached in the summer.

[3] The mortality rates of the industrial policy holders of the Metropolitan Life Insurance Company fluctuate in a manner quite similar to the curves in Text-figs. 8 and 9. In them, however, the high spring peak appears in March and April.

Findings Referable to Different Breeds of Rabbits.

The fact that the rabbits composing this population were originally all pedigreed stock of several definite and well established breeds, and that they have continued to be carefully inbred, afforded a remarkable
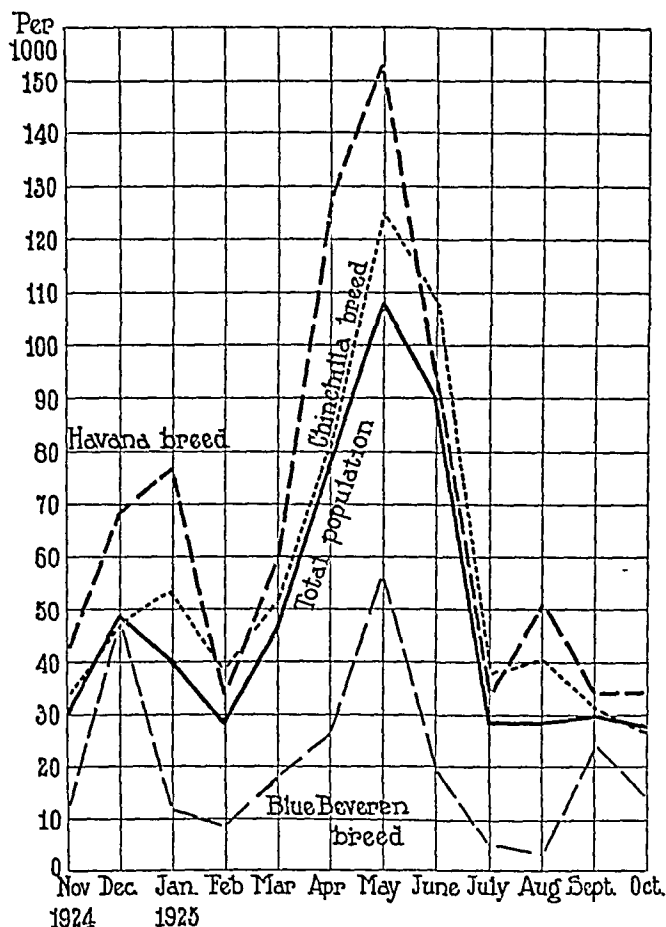
Text-Fig. 12. Comparative mortality rates of Chinchilla, Havana, and Blue Beveren breeds, 1924–25.

opportunity to compare the responses of these different animal breeds to a native disease. *Bact. lepisepticum* infection was widespread throughout the four buildings. Probably, therefore, all breeds were continually exposed to a relatively similar dose of organisms. Likewise, other factors inherent in food and environment which might

affect the behavior of individuals or groups toward a harmful agent were relatively uniform. Hence it appeared not unlikely that under these conditions any differences in the response of one or another breed of rabbits to *Bact. lepisepticum* infection could be attributed to factors inherent in that special breed. Text-figs. 10 to 13 and Tables I to III strongly favor this probability in showing that the various breeds do in fact exhibit consistent and significant differences in mortality, occurrence of snuffles, and *Bact. lepisepticum* carrier rates.
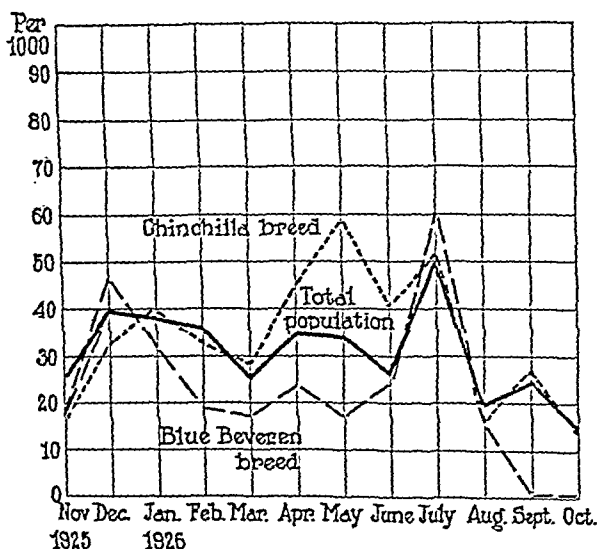
TEXT-FIG. 13. Comparative mortality rates of Chinchilla, Havana, and Blue Beveren breeds, 1925–26.

95 Chinchilla Giants, 158 Belgian hare-Chinchilla crosses, 37 Gaudas, 25 Blue Gaudas, and 14 Blue Flemish animals were studied, but their numbers seemed too small to include in the charts and tables. It is sufficient to state that the snuffles incidence and mortality per cent of the Chinchilla Giants was consistently greater than that of the Chinchillas, that the Belgian hare-Chinchilla crosses were affected to the same degree, and the remainder to a less degree than the Chinchillas.

In Text-fig. 10, the per cent *incidence of clinical snuffles* among about 550 Chinchillas is compared with that of from 82 to 214 Blue Beverens and also with the entire population. Both breeds showed a fluctuating rate which was highest in the spring. Throughout the 10 months period, the per cent of infected Chinchillas was nearly twice that of the Blue Beverens.

Text-fig. 11 shows the incidence of snuffles among 117 Havanas. In this case, the rate rose steadily from the general average to a point higher than that for any other breed.

Total mortality per 1000 among these breeds was observed from November, 1924, to November, 1926. At least 75 per cent was due to *Bact. lepisepticum* infection, hence there are available for comparison 2 years' records of morbidity (Text-figs. 10 and 11) and mortality (Text-figs. 12 and 13) from *Bact. lepisepticum* infection. The curves in Text-figs. 12 and 13, which describe the total mortality of the various breeds, bring out two facts clearly. First, that the seasonal fluctuations in mortality of the entire population are reflected in each separate breed—Chinchillas, Havanas, and Blue Beverens show a spring and fall rise and a summer drop in mortality similar to those of the total rabbit population and the experimental mouse typhoid series. Second, that the mortality among the Blue Beverens was consistently lower than that of the Chinchillas or Havanas. Only once during the 1924–25 period (Text-fig. 12), and three times during 1925–26 (Text-fig. 13) did the death rate of the former equal or exceed that of the latter.[4]

Finally, *the carrier rate* among clinically healthy Chinchillas was compared with that among similar Blue Beverens. The determinations were made as follows.

15 cm. Petri dishes containing 0.5 per cent blood agar were carefully packed and taken by automobile to the New City farm. Sterile swabs were passed into the nares of selected animals and then streaked over the agar surface of the plates. The cultures were then brought back to The Rockefeller Institute, incubated, and studied in the usual manner (1, *b*, *d*). Only rabbits free of nasal discharge and matting of the fur on the fore paws were tested. In all, 142 Chinchillas and 76 Blue Beverens were examined. Table II shows the results.

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[4] The Havana breed, which suffered the greatest mortality, was so reduced in numbers that their record was discontinued in Text-fig. 13.

42 per cent of the Chinchillas and 26 per cent of the Blue Beverens proved to be carriers of *Bact. lepisepticum* on the first examination. A second and third swabbing of the negative Chinchillas increased their carrier rate to 47 per cent and at the same time indicated the usual 5 to 10 per cent error in the results of a single test (1, *b*, *d*). The relative difference between the two carrier rates—Chinchillas 42 per cent, Blue Beverens 26 per cent—is, however, considerable and entirely consistent with the differences between their morbidity and mortality rates.

In brief, these several observations have shown that the rates of snuffles morbidity and mortality among the special breeds of rabbits at the New City farm display the same seasonal fluctuations as do those of the entire population, and that in regard to incidence of

TABLE II.

Carrier Rate of Bact. lepisepticum among the Chinchilla and Blue Beveren Breeds at the New City Rabbit Farm.

Date	Breed	No. of test	No. of animals tested	Carriers of *Bact. lepisepticum*	
				No.	Per cent
April 18–22, 1925....	Chinchilla	1	142	60	42
"	"	3		67	47
May 7–9, 1925........	Blue Beveren	1	76	20	26

Bact. lepisepticum carriers, snuffles cases, and mortality, these breeds show definite and consistent differences.

Findings Referable to Different Individual Animals.

For a period of 10 months, records were kept on each individual adult rabbit at the farm. In November, 1924, February, May, and August, 1925, the general condition of each and the presence or absence of snuffles were noted. During this time many animals died, some of which were autopsied, and a number of replacements were made. All these data are of interest but cannot be included in the present communication. We have therefore summarized the records of 828 animals which had been observed from 7 to 10 months and

have grouped them by means of an arbitrary classification into a table (Table III).

The figures were obtained as follows. Only those animals observed from 7 to 10 months were included under "Total number in selected group." In the third column, the entire mortality among these animals during the 10 months period is recorded. Then from a tabulation of the records of the survivors, the cases were classified as follows, in the order of severity of clinical reaction to *Bact. lepisepticum:* (1) clinical snuffles, 10 months; (2) clinical snuffles at the outset, disappearing and reappearing; (3) clinically normal at first, later developing well marked and persistent snuffles; (4) clinical snuffles at the outset, later disappearing; (5) clinically normal at the outset, developing snuffles which later disappeared; and finally, (6) clinically normal cases throughout the 10 months period. The occasional otitis media, pneumonia, torticollis, ear canker, diarrhea, etc., cases are omitted from this table because they total less than 1 per cent.

TABLE III.

Reactions of Selected Groups of Rabbits to Bact. lepisepticum.

Breed	Total No. in selected group	Mortality (10 mos.)		Clinical snuffles 10 mos.		Clinical snuffles 10 mos., with remissions		Clinically normal at outset, later developing snuffles		Clinical snuffles at outset, later disappearing		Clinically normal at outset, developing snuffles, later disappearing		Clinically normal, 10 mos.	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Chinchillas.. ...	578	286	49.5	59	10.2	13	1.8	88	15.2	40	6.9	25	4.3	67	11.6
Havanas........	144	86	60.0	14	9.7	0	0.0	26	18.5	1	0.7	3	2.1	14	9.8
Blue Beverens....	105	25	23.8	6	5.7	2	1.9	12	11.4	6	5.7	12	11.4	42	40.0

The Havana group showed the most marked reaction to the presence of *Bact. lepisepticum.* 60.0 per cent died, 28.2 per cent had, or developed severe snuffles, and only 9.8 per cent remained clinically normal. Among the Chinchillas as well the effect was severe. 49.5 per cent died, 27.2 per cent showed snuffles during the entire period, or developed it early, and 11.6 per cent appeared clinically normal. The Blue Beverens suffered the least, 40.0 per cent remaining entirely healthy; 19.0 per cent were infected with snuffles at the outset or early during the period, and 23.8 per cent died during the 10 months.

The point deserving special mention, however, is the marked difference in reaction of the individual animals to the presence of *Bact. lepisepticum.* Within each breed there were rabbits which showed no evidence of the disease and others which succumbed; while between these two extremes every gradation was encountered. These differences cannot possibly be due to chance, for it is improbable that the same 67 Chinchillas, the same 14 Havanas, and the same 42 Blue Beverens should have appeared normal on at least four different occasions, over a period of 10 months. Furthermore, the differences in behavior arose under apparently similar environmental conditions and in communities where the bacteria were so widespread that dosage must be considered more or less the same for the entire population. It seems reasonable, therefore, to attribute these variations in animal response, like others which we have noted (1, *g*), to variables inherent in each individual concerned.

DISCUSSION.

The observations on "spontaneous" *Bact. lepisepticum* infection among rabbits have been made under peculiarly advantageous circumstances. Thoroughbred animals, raised in a uniform environment and exposed more or less equally to the risk of infection, comprised the population. Records have been kept of large numbers of individuals over a considerable period of time. The data are therefore relatively free from complicating variables and may be used to advantage in planning and testing the significance of experimental studies.

Investigations dealing with seasonal influences and differences in racial, familial, and individual constitutions show an increasing tendency toward control of complicating variables. Under such circumstances, the more recent findings and interpretations of different observers have been remarkably consistent.

For example, Smiley, in a statistical analysis of the effect of seasonal factors on the incidence of acute respiratory infection, concludes that these infections vary inversely with mean outside temperature and hours of sunlight (4). Chapin, analyzing 102 cases of pneumococcus infection at the Pennsylvania Hospital, notes a relatively high incidence of Type I infection during the winter and early spring months (5). "Common colds" among a group of 6700 clerical employees of

the Metropolitan Life Insurance Company were found to have "two periods of maximum incidence; the first, following the advent of cool weather in the late summer and early fall; the second, occurring during the following January or February when the coldest weather of winter prevails" (6). Schütz in an extensive study of the epidemiology of measles reports that in the large cities of Schleswig-Holstein, the greatest number of cases occur during the spring and fall (7). Pope finds the greatest incidence of scarlet fever to be in January and lowest in July and August but does not think there is evidence of "trend or definite seasonal variation" (8). Rogers attributes the seasonal fluctuations of smallpox in India to the degree of absolute humidity (9). Brown, Pearce, and Van Allen analyzed the first twenty generations of a transplanted rabbit tumor and found that variations in its malignancy tended to follow a seasonal periodicity (10). This they interpreted as due to seasonal fluctuations in animal resistance. Furthermore, by compiling the autopsy data on a large number of so called "normal" rabbits, they found that body and organ weights undergo a definite cyclic change associated with the season (11).

Racial differences in infant mortality in the United States have been studied recently by DePorte. He concludes that the differences in rates of mortality of infants under 1 month of age "very probably have a biological basis and, in this sense, may be termed racial" (12). Meyer and Burghard analyzed the occurrence of scarlet fever and found that family and individual anlages are exhibited definitely by similarity of infection and that the constitution of the individual determines his susceptibility to infection and the course and severity of his disease (13). Lewis (14) and Stevens (15) have noted the same apparent differences in susceptibility to scarlet fever. By means of a skin test with a Shiga dysentery toxin, Brokman and Przesmycki examined 800 individuals for sensitivity. They state that the mechanism of physiological immunity in dysentery is the same as that of diphtheria. It consists in the hereditary capacity to provide normal antitoxin. It is probably a general biological principle which regulates the mechanism of physiological individual immunity against toxogenic bacteria (16). Finally, in a genetic and biometric study of the "Constitutional element in the etiology of pneumonia," Pearl (17) describes a pedigree which has in the father's kinship— "a group of people with a definite tendency towards constitutional inferiority of the respiratory system, which manifests itself chiefly in a tendency to break down from pulmonary tuberculosis in early adult life. This particular constitutional inferiority is absent in the mother's kinship, but in that group of people there is definitely manifest a constitutional tendency to generally non-fatal respiratory infections, bronchitis and bronchopneumonia, in infancy and childhood. When these two constitutional traits were combined, by the mating of the father and mother of the 13 children in the sibship under discussion, there was produced a group of children with extremely low resistance to any sort of respiratory infection, with a consequent 100 per cent incidence of pneumonia in the years of infancy and childhood."

These references to recent literature will perhaps indicate the present trend of thought on these problems. The data are with one exception, which follows, purely descriptive, and have inherent in them a number of indeterminable variables which for the most part have been dealt with according to statistical methods. A direct experimental proof of seasonal variation in mouse typhoid infection has, however, been offered, under conditions in which every known variable except season has been eliminated or controlled (3, *a*). Also racial differences in susceptibility to this infection have been proved experimentally (3, *b*) as well as wide differences in the susceptibility of individuals (1, *h*). Tests with *Bact. lepisepticum* have also furnished evidence that individual rabbits differ in their susceptibility to this disease (1, *i*). Hence the descriptive data are in no small measure supported by the more precise results of direct experiment.

From the general epidemiological view-point, the observations recorded in this paper are of interest in that they describe a rabbit community in which a bacterial infection was endemic and where the type and virulence of the microorganisms concerned were tested from time to time and found to be uniform and constant. A similar study of a rabbit community in which a severe epidemic occurred is reported in the following paper by D. T. Smith (2). Types of *Bact. lepisepticum* recovered by him at Saranac resembled in every respect those occurring at New City. Okamoto, studying epidemics among laboratory animals, found bacterial virulence to be relatively constant (18). R. Freund, investigating a mixed Pasteurella, pneumococcus, and paratyphoid epidemic among a stock of rabbits and guinea pigs at the Robert Koch Institute in Berlin, found no differences in the virulence of various epidemic strains, and concluded that the cause of the outbreaks was due to an increase in population susceptibility following sudden and severe changes in temperature (19). Finally, Theobald Smith and his collaborators have studied a paratyphoid epidemic among a stock of guinea pigs and found no definite fluctuations or differences in the virulence of the specific bacteria. They, too, conclude that changes in population susceptibility must be regarded as the most probable cause of the outbreak (20). Several populations, therefore, have been observed by different investigators to be infected with intestinal and respiratory diseases which have

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undergone epidemic and endemic fluctuations in prevalence and severity without any demonstrable change in the virulence or type of the bacteria concerned.

SUMMARY.

The spread of *Bact. lepisepticum* infection throughout a population of thoroughbred rabbits at a farm in New City, N. Y., was studied over a period of 2 years. The following observations are noteworthy.

1. 50 to 75 per cent of the total mortality was due to *Bact. lepisepticum* infection.

2. About 50 per cent of the stock was affected locally with *Bact. lepisepticum* "snuffles."

3. "Snuffles" and mortality rates showed spring and fall rises and low points during the summer.

4. Carrier, morbidity, and mortality rates of various special breeds differed consistently and to a marked degree. All reflected the same seasonal fluctuations as the total population.

5. Individuals within each breed differed in their response to the presence of *Bact. lepisepticum;* some died of penumonia and septicemia; others developed the local nasal infection, "snuffles;" others became "healthy" carriers; and a few remained uninfected.

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EPIDEMIOLOGICAL STUDIES ON RESPIRATORY INFECTIONS OF THE RABBIT.

X. A Spontaneous Epidemic of Pneumonia and Snuffles Caused by Bacterium lepisepticum Among a Stock of Rabbits at Saranac Lake, N. Y.

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During the winter of 1925, we had an opportunity to observe and study a spontaneous epidemic of *Bact. lepisepticum* pneumonia and snuffles among the stock rabbits at the Saranac Lake Laboratory, N. Y. The circumstances were favorable, since we had some preliminary knowledge, both of the bacteria and of the rabbit populations concerned, and were thus able to determine that throughout the period microbic virulence remained constant and that host susceptibility differences were the predisposing cause of the outbreak. The course of events and descriptive data are given in the following paragraphs.

The Saranac Lake Laboratory for the Study of Tuberculosis maintains a stock of rabbits of mixed Belgian hare breed which had been entirely free from respiratory infection for a period of over 2 years. These animals were either purchased or reared of stock obtained from farmers in the Adirondack Mountains. During the 6 months preceding this epidemic, over 100 rabbits were used by Dr. Willard Soper in a study of experimental tuberculous meningitis (1) and none of the animals developed respiratory infection although the induced disease often ran a chronic course and the infected ones became very weak and much emaciated before death.

At the New York State Hospital for Incipient Tuberculosis at Ray Brook, there existed a stock of rabbits heavily infected with both *Bact. lepisepticum* and *B. bronchisepticus*. In October, 1924, about

fifteen of these rabbits were transferred to the Saranac Lake Laboratory. On arrival, they proved to have snuffles and were therefore isolated from the normal stock. The same attendant fed both the normal Saranac stock and the infected rabbits from Ray Brook, but he did not handle the infected ones or carry them in the baskets used for

TABLE I.

Results of Autopsies on Infected Ray Brook Rabbits before Onset of Epidemic.

No. of rabbit	Presence of snuffles	Bacteriology of nasal passages	Bacteriology of middle ears	
			Right	Left
1	+	*Bact. lep.*	*Bact. lep.*	*Bact. lep.*
2	+	" "	" "	" "
3	+	" "	" "	" "
		B. bronch.		

Bact. lep. = *Bacterium lepisepticum.*
B. bronch. = *Bacillus bronchisepticus.*

TABLE II.

Results of Autopsies on Infected Ray Brook Rabbits after the Onset of the Epidemic.

No. of rabbit	Presence of snuffles	Bacteriology of nasal passages	Bacteriology of middle ears	
			Right	Left
1	+	*Bact. lep.* *B. bronch.*	*Bact. lep.*	*Bact. lep.* *B. bronch.*
2	+	*Bact. lep.* *B. bronch.*	" " *B. bronch.*	0
3	+	*Bact. lep.* *B. bronch.*	*Bact. lep.* *B. bronch.*	*Bact. lep.* *B. bronch.*

Bact. lep. = *Bacterium lepisepticum.*
B. bronch. = *Bacillus bronchisepticus.*

the normal stock. Under these conditions the disease did not spread. A few of the carriers died and were cremated without autopsy, but most of the infected animals remained in excellent condition, as shown by their heavy weight and their smooth, glossy fur.

On February 2, 1925, 5 months later, it was decided to kill the infected animals because they were considered useless for experimental

work. Three of the carriers were autopsied. The sinuses and both middle ear cavities were found filled with pus. Cultures were made on blood agar plates after the method described by Webster (2), and a pure culture of *Bact. lepisepticum* was obtained from the nasal passages and each middle ear of two of the rabbits. From the third animal a pure culture of *Bact. lepisepticum* was found in the middle ears, but in the nasal passages a mixture of *Bact. lepisepticum* and *B. bronchisepticus* was present. Subsequent autopsies and bacteriological cultures on three others showed both organisms in the nose (Tables I and II). It is significant that all six carriers had the form of *Bact. lepisepticum* which Webster (3) has identified as the mucoid type.

Through an error, the carriers were placed in the baskets that were reserved for the normal Saranac stock animals and they remained there for some hours before being transferred back to their cages. The baskets were then used again for the normal animals without being sterilized. Within a week the caretaker noticed that some of the twenty-two normal Saranac stock rabbits were beginning to sneeze. The symptoms of "snuffles," which were mild at first, became more severe and on February 20, 1925, which was 18 days after the accidental contamination of the baskets, the first of the normal rabbits died. The autopsy showed pneumonic consolidation of both lungs and a small amount of seropurulent fluid in the pleural cavities. Cultures and smears of both lungs and pleural fluid revealed *Bact. lepisepticum* in large numbers and in pure culture. During the next 15 days, six other rabbits died of pneumonia. These were all examined, except two, which unfortunately were cremated without autopsy. Those studied showed consolidation of one or both lungs and constant involvement of the pleura. Pure cultures of *Bact. lepisepticum* were found in each case. Some animals had complicating infections of the sinuses and middle ears, while others did not (Table III).

Following this period of high death rate, there was a lull in the epidemic. One animal, which had heavy, labored respiration, continued in that state for 3 weeks and then died. The autopsy revealed a pneumonic process complicated by an empyema on one side only, the other lung being essentially normal. *Bact. lepisepticum* was isolated in pure culture from the right lung, the right pleural cavity, and the right middle ear, while *Bact. lepisepticum* and *B. bronchisepticus* were

TABLE III.

Results of Autopsies on Saranac Rabbits during and after Epidemic.

No of rabbit.	Fate of rabbits	Presence of snuffles	Bacteriology of nasal passages	Bacteriology of middle ears		Bacteriology of lungs	Remarks
				Right	Left		
1	Died	–	–	–	–	Bact. lep.	Pneumonia
2	"	–	–	–	–	"	Pleuropneumonia
3	"	+	Bact. lep.	Bact. lep.	Bact. lep	"	"
4	"	0	0	0	0	"	"
5	"	+	Bact. lep. " " and B. bronch.	Bact. lep.	Bact. lep.	"	Pneumonia and empyema
6	"	+	Bact. lep. and Staph. A.	0	0	0	
7	Killed	+	Bact. lep.	Bact. lep. B. bronch.	Bact. lep.	0	
8	"	+	" " and B. bronch.	Bact. lep. B. bronch.	" " B. bronch.	0	
9	"	+	Bact. lep. and Staph. A.	Bact. lep.	Bact. lep.	0	
10	"	+	Bact. lep.	Bact. lep.	Bact. lep.	0	
11	"	+	"	" "	Bact. lep.	0	
12	"	+	"	" "	"	0	
13	"	+	" " and B. bronch.	" "	0	0	
14	"	+	Bact. lep. and B. bronch.	0	0	0	
15	"	+		0	0	0	
16	"	+	Bact. lep. and B. bronch.	0	0	0	

No.	Killed	+	Bact. lep.				
17	Killed	+	Bact. lep.	0	0	0	Bact. lep. from subcutaneous abscess
18	"	+	"	0	0	0	"
19	"	+	"	0	0	0	Eye infected with B. bronch.
20	"	0	0	0	0	0	Normal.

Bact. lep. = *Bacterium lepisepticum.*
B. bronch. = *Bacillus bronchisepticus.*
Staph. A. = *Staphylococcus albus.*

found in the nasal passages. This was the last of the animals to die of the infection.

At this time the remaining fourteen rabbits were in poor physical condition. They showed marked loss of weight, the hair was rough and untidy, and one had an infection of the eye; another had a large abscess on the buttock, still another had multiple subcutaneous abscesses, and thirteen of the fourteen animals showed clinical evidence of snuffles. The rabbit with the eye infection and the two with abscesses were killed and autopsied. The remaining eleven, under the influence of good diet and care, began to improve rapidly, gained weight, became more active, and within 6 weeks seemed to be normal except for the presence of pus in the nostrils and the matting of the hair on the fore feet. When it became evident that no more rabbits would succumb, the remainder of the group was killed and autopsied, and as shown in Table III, only one of the original twenty-two animals was entirely free from infection at the time of the autopsy, and even this one had clinical snuffles at the height of the epidemic.

Cultures of *Bact. lepisepticum* obtained before, during, and after the epidemic appeared to agree in morphology, colony formation, and cultural reactions. Strains taken from Ray Brook rabbits before the onset of the epidemic and from Saranac rabbits dying of pneumonia during its peak were sent to Dr. Webster at The Rockefeller Institute and found by him to be of the mucoid type, identical with other mucoid forms in cultural reactions and virulence.

SUMMARY.

These observations have brought out three facts which may be stated briefly as follows.

1. An acute respiratory epidemic was incited by mucoid strains of *Bact. lepisepticum* of moderate and uniform virulence.

2. The epidemic was brought about not by an alteration in the virulence of the prevailing strains of *Bact. lepisepticum* but by a sudden distribution of virulent organisms among a susceptible population hitherto unexposed.

3. Individuals in this population showed different degrees of resistance to the infecting bacillus; some died from pneumonia and septicemia, while others localized the infection to the nasal passages.

The significance of these facts has been discussed at length elsewhere (4).

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ON THE COLLECTION OF THE ENTIRE EXTERNAL SECRETION OF THE PANCREAS UNDER STERILE CONDITIONS AND THE FATAL EFFECT OF TOTAL LOSS OF PANCREATIC JUICE.

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Though de Graaf first put a cannula into the pancreatic duct and collected pancreatic juice in 1664 (1), our knowledge of this secretion really dates from the epoch-making discoveries of Claude Bernard (1849) (2), who studied in great detail the juice obtained by placing a silver cannula joined to a rubber tube into the large pancreatic duct. He found, and others after him, that such a method yields pancreatic secretion for only a few days, since infection soon leads to the sloughing of the cannula and the rapid closure of the fistula thus left, the secretion either draining through accessory ducts or newly made channels back to the duodenum.

The successful establishment of a more or less permanent pancreatic fistula was performed by Pavlow (3). Bernstein (4) before him had tried to keep an open channel for the flow of pancreatic juice by inserting lead wires into the duct, but this did not succeed. Pavlow transplanted the entire pancreatic duct together with a rhomboidal piece of duodenal mucous membrane surrounding its orifice into the anterior abdominal wall, closing the buttonhole opening in the duodenum with sutures. Heidenhain described a similar method; but he excised the entire circumference of the bowel, including the opening of the duct, anastomosed the cut intestines, and transplanted the duct as Pavlow did.

In either event, the subsequent course of the operation proved troublesome, for the secretion quickly excoriated the edges of the wound and, even with special precautions, frequently led to the digestion of the anterior abdominal wall (5). Though pancreatic juice, as secreted, has little or no proteolytic power, it was soon demonstrated by one of Pavlow's pupils, Schepowalnikow (6), that the inactive trypsinogen was activated by enterokinase, a secretion of the duodenal mucosa surrounding the duct opening. The difficulty was finally obviated by Babkin (7) who excised the offending duodenal tissue. In this way, a tight scar was formed at the opening of the fistula so that, at times when secretion was desired, a metal or glass catheter was introduced and the juice collected, while at other times it was forced to follow other channels back to the duodenum.

Others have modified the technique of Pavlow and his pupils by isolating the pancreatic duct outside the duodenum, ligating, cutting and sewing the distal end into the anterior abdominal wall. It appears to have been first described by Frouin (8) in 1913 and again by Inlow (9) in 1921, but it was really Senn (10) in 1886 who originally performed this operation and collected pancreatic juice in this way. He was able to collect as much as 120 cc. per day but found that the amount secreted quickly diminished and finally ceased.

Still another type of pancreatic fistula has been described by Fodera (11) who placed a T-tube into the pancreatic duct so that the juice could either go on directly to the duodenum or through one arm of the T to the outside. It is similar in principle to the "amphibilous" biliary fistula described by Schiff (12). But it has never been used by others, apparently, and little is known of its merits.

The general objection, however, to all open fistulas for the collection of pancreatic juice is the impossibility of obtaining sterile secretions. Infection leads to marked alterations of the properties of pancreatic juice and moreover travels up into the ducts and brings about inflammatory changes in the gland itself, and frequently in other organs (13). With an open fistula it is difficult or impossible to prevent licking of the secretions by the animal so that one can only guess how much of the juice is returned to the body and how much is lost.

Similar objections apply to the collection of bile through an open biliary fistula. Rous and McMaster (14) in 1923 described an ingenious operation whereby the total bile was collected under sterile conditions for many months. In our studies of the pancreatic juice we have employed this type of intubation, as described in detail below.

Experimental Procedure.

Under ether anesthesia and with the most scrupulous aseptic precautions, the duodenum was brought into a right rectus incision and the accessory pancreatic duct located on its dorsal side close to where the common duct ends. This duct was ligated and cut and the dissection continued downward till the large duct was reached.

As pointed out by Hess (15), there may be more than two pancreatic ducts in the dog. If there are, they empty into the duodenum between the two largest ones mentioned above. In dissecting the head of the pancreas away from the duodenum, therefore, we were assured of having cut all of them. During the course of our operations, however, we encountered such additional ducts only a few times, and they were very small.

The large duct was dissected from the surrounding tissue and two small arteries which usually accompany it were tied and cut in order to prevent troublesome bleeding later. The duct was then tied close to the duodenum, from the ventral side, a small incision made and the cannula inserted and tied in place. The duct was cut across between the point of intubation and the duodenum. Omentum was tucked between the head of the pancreas and the gut to insure against reconstitution, as well as to cover the raw areas exposed by the dissection.

The collecting tubes were prepared beforehand and autoclaved twice in small Erlenmeyer flasks. They consisted of two parts, one of soft compressible "hemocytometer" tubing (E and A, Fig. 1), the other of harder and firmer material (C and F, Fig. 1). The former was attached to the cannula and, being soft, conformed more or less readily to the intraabdominal movements without kinking, the latter emerged from the abdomen through a separate opening in the side and, being harder, was not so easily compressed. The two were joined by a curved glass connection. The tubes were placed down into the pelvis before
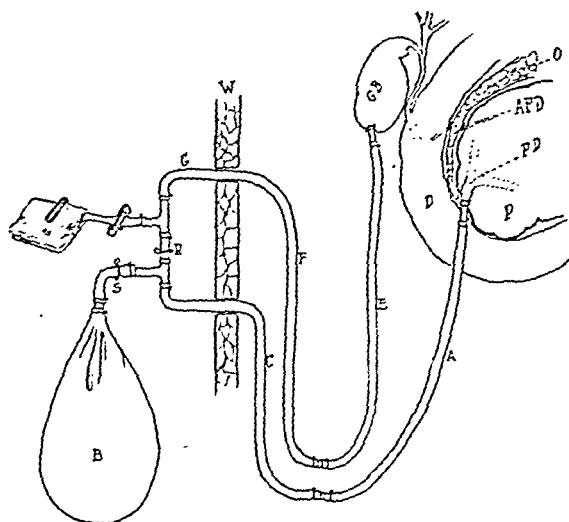
FIG. 1. "Altercursive" intubation of the pancreatic duct. The accessory pancreatic duct (APD) is cut and omentum (O) is tucked between the head of the pancreas (P) and the duodenum (D). Pancreatic juice flows from the main pancreatic duct (PD) through the efferent tube (A–C) into the rubber collecting bag (B) whenever the clamp (R) is closed. When the clamp (S) is closed on the other hand juice flows back through the afferent tube (G–F–E) into the gall bladder (GB) and through the common duct to the duodenum. (See text.)

the abdomen was closed, so that they formed a large U before emerging to the outside. The omentum so adheres to this length of tubing that infection does not extend upward and the collecting system may remain uninfected very often for weeks or months in the absence of other complications.

The emerging tube was attached to a T-tube of glass, the other two arms of which were joined to the collecting balloon and the outlet tube all of which had been previously sterilized by autoclaving. All joints were covered with gauze soaked in 5 per cent phenol.

It was found that the preoperative feeding of the animal was important. All food was withheld for 24 to 48 hours, but, about 5 hours before operation, 200 gm. of finely ground meat was given which acted as a stimulant to pancreatic flow so that when the duct was incised juice poured out profusely thus facilitating intubation and insuring the continuance of secretion afterward without accidental obstruction due to kinking.

Aseptic precautions were maintained particularly during the collection of the juice which was prone to infection by contact or contamination from the air. Collections were made as quickly as possible into sterile containers and the outlet tube carefully covered with several layers of sterile gauze soaked in 5 per cent phenol. The secretion was frequently cultured and the sediment from centrifugalized specimens stained for bacteria. When infection occurred, the animal was either killed or used for other special studies.

EXPERIMENTAL FINDINGS.

We shall here be concerned with those instances in which the flow of pancreatic juice continued uninterruptedly after operation.

In a good many experiments this did not occur. Frequently, the secretion became infected from contact or contamination from the air during periods of collection. In this event, the flow diminished or even ceased entirely in most cases after the lapse of several days. In other instances obstruction developed due, most commonly, to a kink in the rubber collecting tube, a twist in the bag or to the duct's turning on itself so as to close the opening of the cannula. The findings, when such complications took place, proved of much interest and will be described in subsequent reports.

When obstruction or infection did not occur, the secretion continued to flow profusely and, after 5 to 8 days, quite to our surprise, the animals died with marked asthenia, as described briefly in a previous paper (16). In all, twelve dogs were studied in which this fatal outcome was observed.

It is of interest to note that, according to Babkin (5), Bernard, Pavlow, Heidenhain and others observed hypersecretion followed by death in dogs with an open pancreatic fistula. It was regarded as an exceptional occurrence. In our experiments, where the secretion remained sterile and unobstructed, it was the rule.

By the next day after the operation, which usually consumed about an hour, pancreatic juice was found in the collecting balloon and by the 2nd day, the amount had increased. The dog was healthy, active and eating well. Though the secretion was at first slightly turbid, by the

2nd day it had assumed its normal appearance—colorless, very slightly opalescent and quite odorless. It was alkaline enough to turn phenolphthalein a faint pink and when titrated with acid, bubbles of carbon dioxide were given off. With Töpfer's reagent as an indicator, it would neutralize more than its own volume of $N/10$ HCl, the usual value being .11 to .12 N. It contained a large amount of coagulable protein. Amylase and lipase were present in active form. Casein was very slowly digested unless a drop or two of duodenal extract were added when it was dissolved almost instantly.

For 2 days or 3, the animals thus deprived of the total external secretion of the pancreas appeared normal in every way. But then the appetite rapidly diminished. They ate their daily ration of cooked meat, biscuit and milk but instead of eating heartily in gulps as usual, they took longer and often consumed the meal only after several hours or all day. By the 4th day, food was refused entirely and even milk given by gavage was returned. Large amounts of water were taken but soon it was not tolerated. By the 5th or 6th day vomiting had become marked whether fluid was swallowed or not, in the latter case only mucus and froth appearing. The stools, at first bulky and soft, soon diminished in volume and became scanty and loose, although always well pigmented.

Asthenia was noted early and became a prominent symptom. The animal, though in no distress, became increasingly weak and unsteady, reminding one of the asthenia observed after adrenalectomy. But the temperature was not subnormal and respirations and pulse rate were unchanged. By the 6th to 8th day, the animal died. Convulsions were not observed.

The secretion of pancreatic juice continued up to the very end in amounts dependent somewhat upon the size of the dog but apparently in no relation to the intake of food since the largest amounts were secreted in the later days of the experiment when no food was taken at all (see Table I), and while vomiting was persistent. In Dogs VIII and IX, for example, 475 cc. of secretion were secreted during the 24 hours preceding death.

At autopsy, performed in most instances immediately after death, no gross changes were observed. The peritoneal cavity was everywhere smooth and glistening and the operative wound was healed

per primam. The long outlet tube was firmly fixed by omentum and no infection anywhere seen. The pancreas seemed somewhat smaller than normal but was soft and pink. The glass cannula was tightly bound to the duct by fibrous adhesions. The liver and biliary system appeared normal. The stomach was small and firmly contracted particularly the pyloric portion. Its mucous membrane was thrown into deep folds, was hyperemic and showed small erosions or ulcerations. Microscopic sections of the pancreas showed small contracted

TABLE I.

The Amounts of Pancreatic Juice (in C.c) Secreted Each 24 Hours Following Drainage of the Total Secretion, until Death.

Dog No.	Weight	Days								
		1	2	3	4	5	6	7	8	9
	kg.									
I	15	175	275	300	325	325				
II	9	30	170	290	275	250				
III	9	17	185	300	250	185	155			
IV	14	95	260	340	330	440	405	105		
V	16	275	340	245	340	230	245	258	135	
VI	18	25	420	625	590	525	320			
VII	14	275	285	325	460	325	200			
VIII	10	205	280	265	140	315	330	360	475	
IX	11	310	425	205	215	170	210	320	475	
X	10	225	110	200	30	290	300			
XI	14	130	310	300	Obstructed	375	250	125		
XII	12	100	175	210	180	280	305			

acini surrounded by rather wide clear spaces, in distinct contrast to their plump full appearance in the normal gland. The islets of Langerhans seemed unchanged. The adrenals revealed no abnormality.

Blood changes were observed in many cases and will be reported in detail subsequently. In all a marked dehydration was demonstrable by taking hematocrit readings with the Van Allen tube (17). The proportion of red cells to whole blood often reached 80 per cent, the normal being 40 to 50 per cent. There was a decrease in blood chlorides and an increase in pH. Blood sugar determinations were within

normal limits.* The urine was repeatedly examined for reducing bodies but none found.

"Altercursive" Intubation of the Pancreatic Duct.

For the permanent study of the total pancreatic juice, it was necessary to provide for the return of the secretion to the duodenum at will so that the fatal effect of its continued loss might be avoided and still its drainage at times of observation be possible. Such an experimental animal can be prepared by using two tubes instead of one, the second tube being used to return the outflowing secretion through a cannula intubated into the duodenal end of the severed duct.

In the case of bile such a method has already been described by McMaster and Elman (18), and successfully used by them in many experimental studies. The same technique was employed for the pancreatic juice. In this way several dogs were prepared and kept alive for several weeks, and in one case for 2 months, by alternately shifting the secretion to the bag and to the duodenum. If, at any time, the juice were allowed to flow off continuously, the same fatal course followed as described above. On the other hand, two dogs, almost moribund from continued loss of pancreatic juice, were quickly restored to normal by merely turning the flow back to the duodenum.

There are difficulties, however, with the type of altercursive intubation mentioned above, as applied to the pancreatic duct. Frequently, the main duct is so short that there is not enough tissue for the insertion of two cannulas. Attempts to use the accessory duct to return the secretion failed because of the small size of this channel. The most serious obstacle, however, was the difficulty in maintaining sterility of the secretion for very long periods of time. Perhaps it was due to the shortness of the duct which allowed intestinal bacteria to penetrate. In the case of the common duct there appears to be a sufficient protection due probably to its length and the effectiveness of the sphincter of Oddi. Thus, McMaster and Elman (18) found that, with an altercursive intubation of the common duct, the bile remained sterile for months.

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* These determinations were made by Dr. Ronzoni.

It is likely that the difficulty in the case of pancreatic juice lies in the fact that fecal contamination is more readily possible. We have noted that fecal contents are always present in the stomach of dogs draining the total pancreatic juice and it is therefore easy to see how such infective material could easily ascend the short length of duct and reach the collecting tubes.

Another attempt was made by returning the pancreatic juice through the biliary system (see Fig. 1). That is, the tube by which the secretion is to be carried back to the duodenum was inserted into the gall bladder or into the cystic duct after removal of the gall bladder. Two successful instances were possible, no ill effects apparently following the admixture of bile and sterile pancreatic juice. More experiments must be performed, however.

DISCUSSION.

It has been possible by appropriate intubation of the pancreatic duct, by a method described above, to obtain for study the entire external secretion of the pancreas under sterile conditions. The secretion thus obtained is abundant in amount, slightly opalescent in appearance and without odor. It is decidedly alkaline in reaction and contains a large amount of protein, and active lipolytic and amylolytic ferments. Trypsin is present but in the inactive form so that it exerts little proteolytic action. On the addition of a drop or two of duodenal extract it digests protein rapidly.

The fact that such drainage of the total pancreatic juice uniformly leads, in absence of infection or obstruction, to the death of the animal in 5 to 8 days brings up a number of important questions in the mechanism of the external secretion of the pancreas and concerning its relation to the stomach.

The stimulus to this seemingly intense secretion must be one quite apart from the ingestion of food since it continues long after food is refused. The acid gastric juice immediately suggests itself since it is normally a most powerful stimulant of pancreatic secretion. Indeed, we may imagine the gastric acidity as constantly effective under the conditions of the experiment, even in the absence of food, if we assume that the regurgitation of alkaline pancreatic juice into the stomach normally occurs as a regulator of gastric acidity. The drain-

age of the pancreatic juice to the outside thus gives rise to the unopposed acid secretion and a vicious circle is formed quickly leading to the death of the animal since, by the vomiting, the loss of gastric juice is added to that of the pancreatic juice.

From the blood findings mentioned above and from the fact that death occurs on the 6th to 8th day—about the duration of life after acute pyloric obstruction—a possible analogy between the two conditions may exist. The persistent vomiting in itself may be responsible for these findings since Gamble (19) has shown that loss of fixed base in the vomitus brings about the alkalosis and lowered blood chlorides. That the death may be analogous to that following the establishment of a duodenal fistula seems possible. Hartmann (20) has shown that the loss of fixed base in this condition leads to the same blood findings as that following persistent vomiting. The absence of glycosuria and abnormal blood sugar values would seem to rule out any involvement of the internal secretion of the pancreas.

The reason for the persistent vomiting may be connected with the elimination of the regurgitation of the alkaline pancreatic juice into the stomach, which is claimed to be a normal and from our findings might be an indispensable phenomenon. We have frequently noted in our animals that the giving of 0.5 per cent acid solutions into the stomach after the 3rd or 4th day of drainage would promptly result in vomiting even though tap water would be tolerated. Boldyreff (21), in similar experiments noted "the toxic effect of acid solutions in the stomach" of dogs draining pancreatic juice. The prompt restoration of a moribund animal to a practically normal condition by the simple procedure of turning pancreatic juice back into the duodenum suggests the possibility of the presence in the juice of a substance necessary for life.

SUMMARY.

It has been possible by appropriate intubation of the main pancreatic duct of the dog to collect the total external secretion of the pancreas under sterile conditions. When all of the secretion is thus collected exitus occurs in a characteristic way in about a week with anorexia, gastric irritability, vomiting and asthenia. The significance of this finding has been briefly discussed.

A method is also described for the "altercursive" intubation of the pancreas whereby the secretion may be collected or allowed to flow back to the duodenum at will.

To Dr. Evarts A. Graham we wish to express our thanks for his helpful criticisms and advice.

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COMPARATIVE PATHOLOGY OF SOUTH AFRICAN JAGZIEKTE AND MONTANA PROGRESSIVE PNEUMONIA OF SHEEP.

By E. V. COWDRY AND HADLEIGH MARSH.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Montana Livestock Sanitary Board, Helena.)

PLATES 16 TO 18.

Recent studies on a disease of sheep occurring in the northwestern United States, called "progressive pneumonia,"[1,2] appear to have a very definite bearing upon our conception of a South African disease called jagziekte, and *vice versa.* The two conditions have been studied independently, the investigators in each case considering the disease as unique and peculiar to their respective geographical localities. Comparison of publications on the two diseases has brought to our attention the fact that these two chronic pneumonias of sheep, occurring in widely separated parts of the world, are very similar, and apparently are identical in the fundamental characteristics which differentiate them from other described forms of pneumonia. It therefore seems profitable to correlate our knowledge of two such closely related diseases by making a comparative study of the lesions, particularly as in both cases, the diseases cause a considerable loss to the sheep industry, and in neither case has the etiology been definitely established.

Jagziekte has been known in South Africa since 1893. The term is derived from the Dutch *jagt*, to drive, and *ziekte*, a sickness, and is intended to call to mind the fact that the first symptoms appear in animals which are fatigued as a result of having been driven for some distance. The principal lesions are pneumonic and proliferative in character. We have shown the tumor-like proliferations of pulmonary epithelium to several investigators very familiar with cancer. Some

[1] Marsh, H., *J. Am. Vet. Med. Assn.*, 1922–23, lxii, 458.

[2] Marsh, H., *J. Am. Vet. Med. Assn.*, 1923–24, lxiv, 304.

do not hesitate to return a diagnosis of true cancer, while others are more conservative and remark, that, if the same conditions were met with in the human lung, they would classify them as cancerous. No cases have been known to recover. The disease is certainly contagious and is so greatly feared that the farmers immediately kill off all contacts, although they may appear to be entirely healthy.[3]

"Progressive pneumonia" of sheep has been studied in Montana since 1915. Sheep affected with this disease are called "lungers" by the sheepmen. Many sheepmen state that they have known the condition for the last 30 years, but it has apparently increased in recent years and invaded new territory. This is apparently a disease of range sheep as opposed to farm flocks. It is believed that the disease is infectious, and that the infection is probably secondary to mechanical causes. The lesions are similar to those of jagziekte and the prospect of recovery is just as hopeless.

The present study was accordingly commenced in the hope that a detailed comparison of the two conditions might reveal points of correspondence or of divergence, which have hitherto been overlooked, because they have never before been considered together; and, further, that this new information might in turn be of service in unraveling the obscure etiology of both diseases.

Material.

The material upon which this paper is based consists of tissues collected in South Africa from 33 sheep suffering from jagziekte and from 72 control healthy sheep (including some American sheep employed as geographic controls). In addition many clinical cases of jagziekte were observed. The progressive pneumonia material consists of tissues from 46 cases and from 18 supposedly normal sheep. Both histological and bacteriological examinations were made in progressive pneumonia and the symptoms of many typical cases were noted.

OBSERVATIONS.

Before reporting our studies on the comparative pathology of jagziekte and progressive pneumonia, the accompanying table is submitted in order to give a general outline of the two conditions.

Specific reference may be made to one statement in the table; namely, that enclosures in which sheep suffering from jagziekte have been kept constitute sources of infection for incoming healthy sheep, which apparently does not hold

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[3] Mitchell, D. T., *3rd and 4th Rep. Director Vet. Education and Research* (Sir Arnold Theiler), Union of South Africa, 1915, 585.

TABLE I.

	Jagziekte	Progressive pneumonia
Epidemiology	1. Average mortality in infected areas 1.6 per cent *per annum*	1. About 2 per cent *per annum* in infected bands
	2. Occurs throughout the year	2. The same
	3. All ages affected, but chiefly at 3 yrs.; never lambs	3. Most of the deaths occur in sheep 4 yrs. old and over; but many clinical cases have been observed in 2 yr. olds, a few in yearlings, and early stages have been seen in lambs on postmortem examination
	4. Occurs in both range sheep and farm flocks, but chiefly the former	4. Occurs in range sheep, but not in farm flocks
	5. High altitude and low humidity	5. The same
	6. High mean annual temperature	6. Lower mean annual temperature
	7. No evidence that the character of water supply and of the grazing are determinative factors	7. The same
	8. Apparently transmitted by contact, since it appears after importation of new sheep	8. The same
	9. Enclosures in which cases of jagziekte have been kept are said to constitute sources of infection for healthy sheep for 10 days or more after the diseased sheep have been removed	9. Not known to be the case
Symptoms	1. Increasing respiratory distress with catarrhal nasal discharge	1. Increasing respiratory distress, with but little nasal discharge in most cases
	2. Extreme emaciation	2. The same
	3. Little or no increase in temperature. Failure to note a temperature reaction may be due to the fact that the sheep are seldom observed clinically in the earliest stages of the disease, which are in fact not detectable	3. The same

TABLE I—*Continued.*

	Jagziekte	Progressive pneumonia
Symptoms—*continued*	4. The disease extends over a period of several mos. At the outset destructive lesions may exist which are not revealed by clinical symptoms	4. The disease extends over a period of several mos. after symptoms appear. The period during which it exists, but cannot be detected clinically, may extend for several mos. or even yrs.
Prognosis	Duration of life may be prolonged by careful treatment, but it is said to be invariably fatal.[3] There is, however, some evidence that animals in the earliest stages may recover[4]	The same
Prophylaxis	Immediate slaughter of all contacts and the abandonment or careful cleaning of quarters occupied by sick sheep	Slaughter of all recognizable cases
Etiology	1. No organisms have thus far been constantly observed in the specific lesions, although bacteria of several kinds are frequently present, usually in association with inhaled dust-like foreign material	1. Two bacteria have been constantly isolated in cultures made from the lungs, one or both of which may be the causative agents[2]
	2. Eosinophil leucocytes, which one would expect in nematode infestations, are not unusually abundant	2. The same
	3. Poor general condition and insufficient food are not of primary importance as predisposing factors	3. The same
	4. It has never been possible to transmit the disease by the inoculation of material of any kind from diseased animals	4. Repeated intratracheal inoculations of a diphtheroid from affected sheep produced small lesions of the same character as the extensive lesions found in naturally diseased animals. One sheep which was inoculated intratracheally with lung tissue developed the disease

TABLE I—*Concluded.*

	Jagziekte	Progressive pneumonia
Etiology— *continued*	5. The intratesticular and intra-cerebral inoculation of tis-sue—procedures indicated by the tumor-like appear-ance of the epithelial growth—have not been at-tempted	5. The same
Pathology	1. The specific lesions are re-stricted to the thoracic cavity	1. The same
	2. The primary changes are found in the interalveolar tissue of the lungs. They occur in foci several mm. in diameter and consist of engorgement of the alveolar capillaries and of the inter-stitial accumulation of mac-rophages and lymphocytes. Frequently they are associ-ated topographically with deposits of above mentioned foreign material[4]	2. The same, except that this primary interalveolar in-filtration seems to be more extensive
	3. Exudation of macrophages and of leucocytes then takes place into the alveoli re-sulting in a typical chronic catarrhal pneumonia. At the same time there is a marked and very luxuriant tumor-like proliferation of the epithelial cells of the alveoli and bronchioles[5]	3. The same, except that the epithelial proliferation is usually less pronounced and resembles more a benign hyperplasia. The growth is distinctly less invasive and less neoplastic in ap-pearance. Dividing epi-thelial cells are more abun-dant, also alveolar casts
	4. There is sometimes a slight tendency toward localized resolution of the pneumonic process, but this is rare and never takes place on a large scale	4. Not observed
	5. The blood supply is reduced and extensive fibrosis takes place. The animals appar-ently die from asphyxiation through reduction in re-spiratory area	5. The same

in progressive pneumonia. It is based upon casual observation only and is difficult to prove, because it has been found that jagziekte, in its earliest stages, is not recognizable clinically[3,4] so that supposedly healthy sheep employed in the experiments may not be in reality free from the disease to begin with.

Examination of gross specimens shows that the lesions commence in definite foci and spread thence throughout the pulmonary tissue. In the more central parts of the affected areas the lesions are older, and fibrosis and consolidation have frequently set in. It is possible, therefore, by selecting specimens for histological examination, first remote from the principal lesions and then nearer and nearer to them, to secure a series of preparations which give some idea of the sequence of changes involved. The earliest changes may, we believe, occasionally also be found by the examination of sheep which have as yet shown no clinical signs of the disease, but which come from affected flocks, through a comparison of their tissues with those of sheep known to be free from both diseases.[4] Lungs from cases of jagziekte of this kind show the infiltrative, exudative and slightly proliferative changes which we are about to describe, uncomplicated by the pneumonia and fibrosis characteristic of more advanced stages.

In the plates illustrating the lesions, the photomicrographs on the left are from cases of jagziekte and those on the right from cases of progressive pneumonia. For additional illustrations of the diseases considered separately, reference may be made to earlier publications.[1-5]

The Interalveolar Tissue.

In both diseases the primary changes center in the interalveolar tissue, which is thickened (Figs. 1 and 2). They are identical in quality but seem to be rather more extensive in progressive pneumonia.

The thickenings are caused principally by accumulations of lymphocytes and large mononuclear cells which have been variously called macrophages (Metchnikoff), endothelial leucocytes (Mallory) and polyblasts (Maximow). None of them exhibits signs of mitotic division. Sometimes, in both conditions, the lymphocytes may greatly outnumber the large mononuclears and give rise to nodules

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[4] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 323.

[5] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 335.

recognizable in gross specimens. The nodules are often peribronchial in position. Both of these cells are accompanied by only a few polymorphonuclear leucocytes. Eosinophil leucocytes are not unusually abundant either in the lesions or in the circulating blood.

The exact source of these large mononuclears is difficult to ascertain, although they are probably chiefly of hematogenous origin. No evidence was found that they are produced through the multiplication of the endothelial cells of the alveolar capillaries as claimed by Permar.[6] In some jagziekte preparations they were seen within the capillary lumina in contact with erythrocytes and free from the lining endothelium (foot-note 4, Fig. 9). In all likelihood they may also arise from cells resident in the interalveolar tissue; in other words, from the rhagiocrine cells of Renaut, or the histiocytes of Kiyono (to employ only a few of the available synonyms). That they may be derived from the lymphocytes which are likewise present normally in small numbers in the interalveolar tissue is to be considered as a third possibility in view of Maximow's[7] observation that freshly emigrated lymphocytes are capable of transformation into large phagocytic ameboid cells; that is to say, into the mononuclear cells. The lymphocytes, in turn, may emigrate from the alveolar capillaries, since, although we have no specific information regarding the pulmonary lymphatics of sheep, Miller[8] was unable to find in man any lymphatics in the walls of the air sacs beyond the ductuli alveolares.

The thickenings in jagziekte frequently also contain, in addition to the cells enumerated, deposits of inhaled foreign material which have been already reported,[4] and there is reason to believe that in progressive pneumonia substances of this kind may likewise act as predisposing factors.

With the foreign material in jagziekte, bacteria of several kinds are frequently noted on microscopic examination, and it is probable that cultural methods would bring to light the existence of a varied bacterial flora. Several bacterial invaders are probably also concerned in progressive pneumonia, the bacteriology of which has been much more systematically studied.[2]

The fact that Marsh succeeded in experimentally producing small lesions, like those of typical cases of progressive pneumonia, by the intratracheal inoculation of a diphtheroid isolated from affected sheep

[6] Permar, H. H., *J. Med. Research.*, 1920–21, xlii, 147, Plate IV.

[7] Maximow, A. A. *Physiol. Rev.*, 1924, iv, 533.

[8] Miller, W. S., *Am. Rev. Tuberc.*, 1919–20, iii, 193.

is suggestive, especially in view of some recent experiments by Grum-
bach,[9] who, by the injection, similarly of a diphtheroid ("*Coryne-
bacillus diphtheroide*"), caused pulmonary lesions in guinea pigs,
which he regards as almost identical with those of jagziekte in sheep.
Grumbach has very courteously sent microscopic preparations to us
and we have verified the close resemblance which some of them bear
to jagziekte and progressive pneumonia.

Coincident with these interalveolar infiltrations in both diseases,
there is often a noticeable localized vascular engorgement.

The Alveolar Exudate.

This consists chiefly of large mononuclear cells which are like those
already mentioned in the interalveolar tissue but are modified to some
extent owing to enhanced phagocytic activity. Their appearance
is the same in progressive pneumonia as in jagziekte in which they
have been described in detail in a previous paper[4] which is supple-
mented by several photomicrographs. Attention may however be
directed to their properties, as represented in the lower right hand
corner of Fig. 8. There may be some polymorphonuclear leucocytes
in addition, but the latter do not as a rule predominate in either
jagziekte or progressive pneumonia except in later stages, when an
acute pneumonic process is often met with (Figs. 11 and 12). Poly-
nuclear giant cells, arising from the mononuclears, are often en-
countered. They are illustrated in Fig. 11 among the leucocytes.
There is some desquamation of the epithelial cells. Coagulated blood
protein is not abundant in the exudate.

The nature of the mononuclear cells, which are frequently known as "epithelioid
cells" and "dust cells," is difficult to determine. Some investigators[10-13] believe
them to be epithelial cells which have hypertrophied and desquamated into the
alveolar lumina. Others incline to the view that they are of hematogenous origin.
Permar[6] stimulated the production of these cells by injecting into the air spaces
finely divided foreign material and followed all stages in their migration, having

[9] Grumbach, A., *Bull. Assn. franç. étude cancer*, 1926, xv, 213.

[10] Briscoe, J. C., *J. Path. and Bact.*, 1908, xii, 66.

[11] Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.

[12] M'Fadyean, Sir John, *J. Comp. Path. and Therap.*, 1920, xxxiii, pt. 1, 1.

[13] Mavrogordato, A., *J. Hyg.*, 1918, xvii, 439.

first marked them by vital staining with pyrrhol blue. He found no evidence that the nucleated or non-nucleated respiratory epithelial cells, described by Ogawa,[14] took up the dye. Lewis, Willis and Lewis[15] closely correlated the types of cells observed in experimental pulmonary tuberculosis as seen in sections and with the aid of supravital stains. They discovered that the epithelioid cells, both in the tubercles and within the lumen of the alveoli, resembled the large mononuclears (macrophages or monocytes) of the blood in many details of their structure (nuclei, centrosomes, mitochondria, neutral red granules) as well as in their phagocytic properties.

In jagziekte we believe that these large mononuclear cells may in the vast majority of cases be distinguished from desquamated respiratory epithelial cells by the following criteria:

1. Their nuclei are often kidney-shaped (and may thus be contrasted with the spherical nuclei of the epithelial cells) and by staining with iron-hematoxylin a centrosome, or a diplosome, is revealed on the side of the nuclear concavity.

2. The centrosome is always centrally placed (remote from the periphery of the cell) and the cytoplasmic granules about it are arranged radially. In hematoxylin and eosin preparations the position of the centrosome is marked by an area of cytoplasm which stains less intensely. Methods for the supravital staining of blood elements[16, 17] were not employed and it is doubtful whether they would be helpful in this case, because the question of the source of the cells thus examined (*i.e.* from the alveolar lumina, or ruptured alveolar capillaries, or interalveolar tissue) would remain.

3. There are many rod-like mitochondria in the cytoplasm accompanied by droplets of fat which may be colored with Sudan III. Both of these components are difficult to find in unaltered epithelial cells.

4. The mononuclear cells are often present in such enormous numbers as to suggest some almost inexhaustible reservoir of origin, such as the blood stream, as contrasted with the limited surface of the alveolar walls.

5. They are actively phagocytic for just those substances which macrophages are known to take up, and, on general principles, it would seem unlikely that desquamated epithelial cells would exhibit this property. In all other parts of the body desquamating epithelial cells are either dead or dying.

6. They appear within the alveoli before the epithelial cells hypertrophy and multiply *in situ*, or extend over the internal alveolar surface from restricted clusters, if we accept Ogawa's conclusion regarding their normal distribution.

7. What appear to be actual stages in the entry of these mononuclears into the

[14] Ogawa, C., *Am. J. Anat.*, 1920, xxvii, 333.

[15] Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

[16] Cowdry, E. V., *Internat. Monatschr. Anat. u. Physiol.*, 1913–14, xxxi, 267.

[17] Simpson, M. E., *Univ. Calif. Pub. Anat.*, 1921, i, 1.

alveolar lumina, may in rare instances be seen and identified by their position and the hour-glass-like shape of their nuclei, when they are fixed half inside and half outside the wall of the alveolus (foot-note 4, Fig. 10).

8. In more advanced stages of the disease, when the epithelial proliferation has become extensive, so that all the alveoli are lined by cubical or columnar cells, the number of large mononuclears within the alveolar lumina is markedly diminished (see Figs. 5 and 6). This decrease in the mononuclears as far as can be determined is coincident, or follows immediately after, a constriction of the blood vessels caused by the pressure of the proliferating epithelial tissue and by interalveolar fibrosis. If the mononuclear cells were produced, for the most part, by the desquamation of epithelial cells, one would expect them to appear when the epithelial cells are most abundant instead of at a time when few if any of them may be seen and none of them has undergone any noticeable preparatory hypertrophy.

9. Some epithelial cells do, however, unquestionably desquamate. They break away from the alveolar wall in groups, seldom singly, by the operation of some factor or factors which inhibit or destroy their cohesive properties. Frequently the entire hypertrophied layer of alveolar epithelium splits away from the wall of the alveolus and comes to occupy a position within it as indicated in Fig. 7 of jagziekte and in Fig. 8 of progressive pneumonia.

The origin of most of the mononuclear cells seems, thus, to be the same—namely, from the contents of the alveolar capillaries and the interalveolar tissue—in both jagziekte and progressive pneumonia, although we have not examined the centrosomes, mitochondria and neutral fat in the latter condition.

The Epithelial Proliferations.

There is some question whether distinctive proliferations of the respiratory epithelium are to be observed in all cases. While Theiler[18] considers them to be definitely indicative of jagziekte, Mitchell[3] failed to find them in two out of fifteen cases and did not refer either to their presence or absence in three others. In progressive pneumonia Marsh has not always found them. In general they seem to be of slightly more constant occurrence in jagziekte.

In both conditions they commence in isolated foci like those represented in Figs. 2 and 3. That they originate through a metamorphosis of alveolar epithelium may be readily ascertained by the study of serial sections, which shows that they are often entirely separate and apart from the epithelium of the bronchioles. But the bronchiolar

[18] Theiler, Sir Arnold, personal communication.

epithelium also proliferates, though less extensively. These foci of overgrowth of the alveolar epithelium later merge with the result that large masses of tissue become almost (or in fact) adenomatous (Figs. 5 and 6) and present an appearance resembling superficially a mammary gland regenerating during lactation. The cells are sometimes, in the older growths, quite atypical in their properties (foot-note 5, Fig. 12). They are generally arranged in a single layer but in both diseases many of them may become superposed so that irregular masses of cells result. Within the bronchiolar proliferations dense and circumscribed clumps of polymorphonuclear leucocytes, each from about ten to twenty cells in number, may occasionally be distinguished.

Mitotic figures are of comparatively rare occurrence but appear to be a little more numerous in progressive pneumonia. Nuclear appearances indicative of widespread amitotic multiplication of the epithelial cells were seldom observed in either disease.

In general, the proliferations seem to be somewhat more luxuriant and tumor-like in jagziekte, but cases of progressive pneumonia are often observed in which they are equally highly developed and constitute just as conspicuous features of the disease. They are slightly invasive in both (foot-note 5, Fig. 11), but never metastasize, even to lymph glands within the thorax. Other parts of the body are not affected except by the general cachexia.

The proliferations are obviously secondary to the infiltrative and exudative changes already referred to. In common with hyperplasias of respiratory epithelium in man[19,20] they constitute a sort of aftermath of a preexisting infection. But they are undoubtedly more marked than any which have been reported in man, except in the case of definite neoplasms in which, however, the distinctive and primary interalveolar changes, already referred to, are generally absent.

Other Lesions.

A comparison of Fig. 7 of jagziekte and Fig. 8 of progressive pneumonia with Figs. 5 and 6 of earlier lesions will illustrate the commenc-

[19] Winternitz, M. C., Mason, I. M., and McNamara, F. P., The pathology of influenza, New Haven, 1920.

[20] Hart, C., *Deutsch. Arch. klin. Med.*, 1904, lxxix, 108 (measles).

ing interalveolar fibrosis which becomes very marked in advanced stages of both diseases and leads to widespread consolidation. The occurrence of small definitely outlined areas of myxomatous tissue is also typical (Figs. 9 and 10).

The pneumonic process is, as far as we can ascertain, of fundamentally similar nature in jagziekte and progressive pneumonia. Corresponding parts of the lungs are affected and it spreads in the same way, bringing about eventually the death of the animal.

Comparison of Jagziekte and Progressive Pneumonia with Verminous Pneumonia.

Through the kindness of Sir John M'Fadyean we have had the privilege of examining also a specimen from a case of verminous pneumonia in sheep. On the basis of this specimen and of his published description[12] it would apparently be difficult, through microscopic examination alone, to distinguish the lesions of this disease from those of jagziekte, unless perhaps very abundant material were available. Like jagziekte, the lesions of verminous pneumonia differ from those of progressive pneumonia chiefly by somewhat lesser involvement of the interalveolar tissue and by slightly more intense epithelial proliferation. The presence of nematodes and of their ova within the lungs in verminous pneumonia and their undoubted rôle in the production of the disease, as discovered by M'Fadyean, led us to make a very careful search for animal parasites in both jagziekte and progressive pneumonia, which was, however, unavailing.

DISCUSSION.

The pathogenesis of the lesions which we have briefly described in jagziekte and progressive pneumonia suggests the conclusion that the lungs have been subjected over a rather long period of time (4 to 8 months) to a variety of injurious influences, mechanical, in the form of foreign material, and infective, through bacterial invasion. It seems indeed surprising that similar conditions have not been reported from still other localities. With the possibility in mind that, although such accounts have not been published, the diseases may nevertheless occur, letters of inquiry were sent to Professor José Lignieres at Buenos Aires (Argentina) and to Professor Harold A. Woodruff at Melbourne (Australia). The answers received, together with a letter from Dr. H. R. Seddon of the Department of Agriculture, New South Wales, indicate that no cases of disease resembling jagziekte

or progressive pneumonia have thus far attracted attention in these great sheep-raising countries.

Definite predisposing influences are unknown, but that a dietary factor may nevertheless be involved is indicated by Theiler's study of jagziekte in horses,[21] which he found to be caused by the eating of a poisonous plant (*Crotalaria dura*), but as yet there are no observations available which point in this direction in the case of these two destructive diseases of sheep. Nor do we know just how closely jagziekte of horses resembles jagziekte of sheep. From Theiler's description it would appear that the epithelial proliferations are alike; but it is no longer possible to collect material for comparison without experimentally producing the disease by feeding; because as soon as its cause was discovered, preventive measures were taken which have completely banished it from the Union of South Africa.

In order to secure advice as to whether the action of some foreign protein might, in fact, constitute a specific predisposing factor in jagziekte, specimens were sent to Dr. C. F. Hoover of Western Reserve University. Dr. Hoover states that it is conceivable that repeated inhalations of protein may be responsible for such a chronic condition, but that if this were the case one would expect the attacks of respiratory distress to be paroxysmal, which is not characteristically so in either jagziekte or progressive pneumonia.

In regard to the precise cause of the epithelial proliferations, very little may be said. Robertson's[22] theory that they are produced by a malaria-like microorganism has received no support. It seems likely, when we take into consideration the occurrence of similar proliferations in verminous pneumonia,[12, 23] and the multiple adenomata reported by Ebner,[24] that the respiratory epithelium of sheep is unusually prone to undergo this change. That epithelial proliferations are commonly produced by bacterial infections[25] and by a variety of experimental agencies[26] is well known. Yet as far as our

[21] Theiler, Sir Arnold, *7th and 8th Rep. Director Vet. Education and Research*, Union of South Africa, 1918, 59.

[22] Robertson, W., *J. Comp. Path. and Therap.*, 1904, xvii, 214.

[23] Hofman, V., *Časop. lék. česk.*, 1923, lxii, 65; abstracted in *J. Am. Med. Assn.*, 1923, lxxx, 1279.

[24] Ebner, A., *Z. Tiermed.*, 1899, iii, 161.

[25] Teutschlaender, *Centr. allg. Path. u. path. Anat.*, 1914, xxv, 424.

[26] Fischer, B., *Frankf. Z. Path.*, 1922, xxvii, 98.

present information concerning jagziekte is concerned it is still possible that the proliferations may arise through the action of a single ultravisible virus, as claimed by Theiler;[18] but we do not believe that the entire disease complex is caused by such a virus. It is interesting, in this connection, to recall that Roux[27] discovered the existence of an adenomatous proliferation of the respiratory epithelium in sheep-pox, but unfortunately he only referred to it very briefly and gave no illustrations.

If the proliferations in jagziekte and progressive pneumonia are in truth neoplastic, it is difficult to explain Mitchell's failure to transmit the disease by the inoculation of tissue fragments despite the fact that he did not avail himself of recent methods of intratesticular inoculation with a large series of animals. Perhaps the failure is due to the difficulty of producing experimentally the primary lesions, the nature of which we have stressed and which may be prerequisite to the growth of the tumor. Acceptance of the tumor hypothesis would render intelligible the fatal outcome in all recognizable cases of the diseases. Obviously, if we are dealing with a tumor, it does not mean that we are faced by one which of itself and independently spreads from diseased to healthy sheep; on the contrary, the view that we have to do with a neoplasm which comes in the wake of an acute infection or series of infections, most probably bacterial in nature, is not improbable.

SUMMARY.

As long as the exact etiology of jagziekte and of progressive pneumonia remains unknown, it cannot be said that they are identical, although on the basis of the observations detailed above, they certainly appear to be. It is extremely doubtful whether it is possible to distinguish between them. In the localities in which they occur, each is recognized as an infection quite distinct and apart from other better known pneumonias. Predisposing factors which lead to bacterial invasion are presumably of great importance in both. We have no precise information regarding the organisms concerned, but in both diseases, the initial changes are alike and occur in the interalveolar tissue and the subsequent proliferations of epithelium and the pneu-

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[27] Roux, E., *Bull. Inst. Pasteur*, 1903, i, 49.

monia are of the same character and apparently follow in exactly the same sequence. Finally, in both diseases, there is much fibrosis and the animals inevitably die through depletion of respiratory area and pneumonia.

The view hitherto held, that jagziekte is a disease definitely restricted to the Union of South Africa, is thus rendered improbable. Nor does it appear likely that jagziekte is due to a single specific virus acting primarily upon the epithelial cells of the lungs, which is is likewise a conception widely accepted in South Africa.

EXPLANATION OF PLATES.

To facilitate comparison of the two diseases photomicrographs taken at a magnification of 240 diameters of approximately similar lesions are given in pairs, jagziekte on the left and progressive pneumonia on the right hand side.

PLATE 16.

FIG. 1. Interalveolar infiltration in jagziekte.

FIG. 2. The same in progressive pneumonia.

FIG. 3. Beginning epithelial proliferation in jagziekte.

FIG. 4. The same in progressive pneumonia with rather more interalveolar infiltration.

PLATE 17.

FIG. 5. More advanced proliferation in jagziekte.

FIG. 6. The same in progressive pneumonia.

FIG. 7. Desquamation of proliferated epithelium in jagziekte.

FIG. 8. Same in progressive pneumonia with less interalveolar change. The alveoli in the right lower corner contain typical large mononuclear cells.

PLATE 18.

FIG. 9. Nodule of myxomatous tissue in jagziekte.

FIG. 10. The same in progressive pneumonia. It will be noted that the cells are larger because they have not been shrunken so greatly by the fixative.

FIG. 11. Masses of polymorphonuclear leucocytes in the lumina of bronchioles in jagziekte. Many polynuclear giant cells are visible.

FIG. 12. The same in progressive pneumonia except that in this case the proliferated epithelium is rather lower, being more typical of the altered alveoli, and giant cells may be only indistinctly seen.
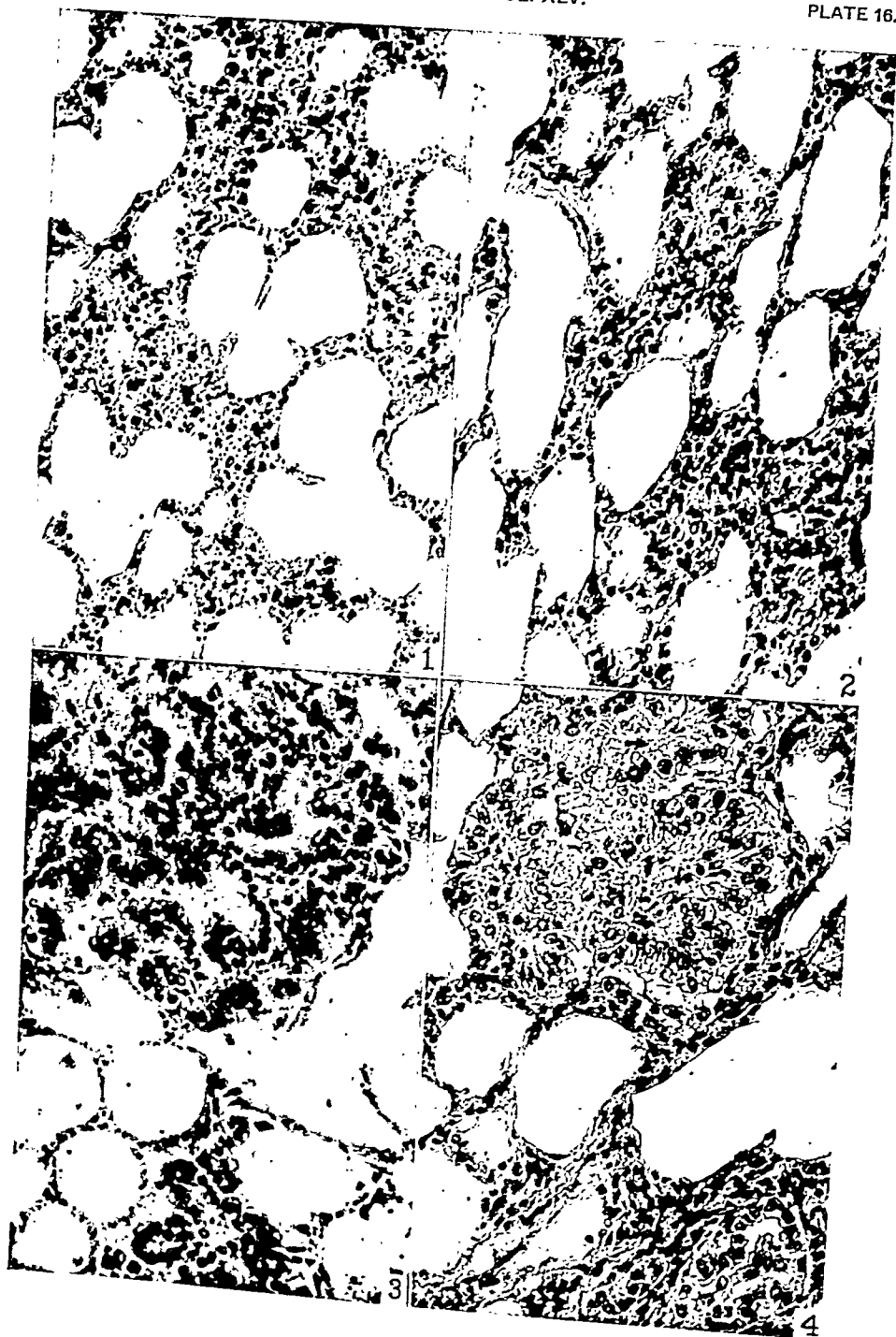
(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)

ON THE TRANSPLANTATION OF THE GUINEA PIG SUPRARENAL AND THE FUNCTIONING OF THE GRAFTS.

By HENRY L. JAFFE, M.D.

WITH THE ASSISTANCE OF ALMA ADLER.

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PLATE 19.

In 1906 Elliott and Tuckett[1] reported their inability to transplant the guinea pig suprarenal homoplastically into the subcutaneous tissues of the abdominal wall. Within a few hours after grafting a half gland beneath the abdominal skin of a guinea pig the surrounding tissues swelled and appeared irritated. Next day the swelling increased, the skin over the transplant turned green, and a foul smelling, clear fluid transuded from between the skin sutures. The escape of the fluid relieved the irritation, the skin over the swollen area sloughed away, and the animal recovered. If the fluid did not escape, the irritation extended rapidly over the surface of the body, the animal's temperature fell, and it became comatose and died in 30 hours. The only organ showing noteworthy pathology was the suprarenal, the cortex of which was congested, hemorrhagic, and without the brown granules, while the medulla showed the absence of chrome staining.

To our knowledge the successful transplantation of the guinea pig suprarenal has not been reported, due, we felt, to the failure to remove the medullary tissue from the grafts. Taking this precaution we sought to obtain positive and functioning autoplastic transplants in this animal. All previous experience has shown that medullary tissue never regenerates, so that its removal is of no consequence in a study of suprarenal transplants.

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[1] Elliott, T. R., and Tuckett, I., Cortex and medulla in the suprarenal glands, *J. Physiol.*, 1906, xxxiv, 361.

Methods.

Most of the guinea pigs used were born in the laboratory, and were between 2 and 3 months old at the beginning of the experiment, except for a few aged 5 or 6 months. Under ether anesthesia first the right suprarenal was completely removed through the dorsal route, and was kept in normal saline at 38°C. until the wound was closed. It was placed on a cork and cut longitudinally with a sharp razor blade into 4 or 8 segments. The medulla together with some of the adjacent cortex was removed from each segment with fine curved dissecting scissors, to insure extirpation of the epinephrine-containing tissues. Each segment of cortex was divided transversely, and the small pieces were washed for 15 minutes in the physiological saline. Meanwhile many pockets were prepared in the abdominal wall by puncturing the muscle with a cataract knife, and enlarging the openings by spreading an ordinary forceps in them. One fragment of suprarenal cortex was introduced into each pocket, the mouth of which was closed with a black silk marking suture. The skin was sewed with silk and covered with celloidin. 3 to 7 weeks later the left suprarenal was removed. All tissues were fixed in Zenker-formol, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin.

Data.

In 22 guinea pigs the right suprarenal was removed and autotransplanted into the abdominal muscle. 3 to 7 weeks later the left suprarenal was removed from 17 of these. Some animals died from operative shock, others from suprarenal insufficiency, and still others were killed in good condition to terminate the experiment. There were no toxic deaths attributable to the transplants, although irritation and swelling of the abdominal muscle frequently occurred. 84 per cent of our animals had 1 or more takes varying from nests of a few cells to masses several mm. in diameter when examined after 3 weeks. Although 8 to 16 transplants were inserted in each animal, at autopsy usually only 3 or 4 were found. In the guinea pig the percentage of transplants that take compared with the number of transplants inserted is quite small because the severity of the reaction following the transplantation influences the course of the regeneration.

In spite of the gaps in our series, the successive histological changes in guinea pig suprarenal transplants can be followed. Stages of degeneration and regeneration, followed either by the absorption of the regenerated transplant or its marked growth, have been observed.

After 3 days transplants show marked necrosis, only a few supra-

renal cells remaining at the periphery. These surviving cells show cloudy protoplasm with indistinct cell walls. The necrotic area is edematous and is infiltrated by some polymorphonuclear leucocytes and round cells. At the periphery of the transplant new blood vessels have appeared. About the transplant the tissue has become edematous, some polymorphonuclear leucocytes and round cells have infiltrated, and sprouting fibroblasts appear.

By 11 days the transplant area is very celullar with little debris remaining. Great numbers of polyhedral cells are present having large vesicular nuclei, prominent nucleoli, and hazy, granular, pinkish staining cytoplasm. These cells sometimes occur in small groups appearing like multinuclear giant cells. Connective tissue cells are seen between the regenerating suprarenal cells, and new blood vessels penetrate the transplant. Some of the regenerating suprarenal cells are dividing mitotically. The muscle about the transplant shows an intense interstitial inflammation, and muscle giant cells and pigmented cells are seen.

By the 22nd day the transplants diminish in size and appear macroscopically as tiny yellow-white specks which fuse in color with the fascia of the abdominal muscle. Microscopically groups of newly regenerated suprarenal cortical cells in glomerular formation are seen. In addition considerable connective tissue has formed between these cells. Large numbers of lymphocytes appear in some transplants at this stage, probably in those destined for rapid absorption.

During the 4th and 5th weeks regeneration is active, and the suprarenal cells increase by hypertrophy and hyperplasia, and glomerular and fascicular formations appear. The transplant areas still show considerable interstitial connective tissue. At about this time transplants show tendencies either to absorption or rapid growth. Absorption progresses slowly, taking many weeks, and is characterized by shrinking of the suprarenal cells, progressive lymphocytic infiltration and interstitial fibrosis, and the appearance of many pigmented phagocytes. During the course of this slow absorption many of the animals die of suprarenal insufficiency, evidently due to the inability of the transplants to maintain life.

On the other hand the transplants which show active growth enlarge by the multiplication of suprarenal cells mostly through amitotic

division while the interstitial connective tissue diminishes. Dark, actively growing suprarenal cells are mingled with lighter larger cells, and as regeneration proceeds the cells become larger and more fatty. After 100 days some transplants show the glomerular, fascicular, and reticular layers while the connective tissue and lymphocytes have disappeared. With further growth the transplants sometimes appear as masses of very large closely packed polyhedral cells with markedly vacuolized protoplasm. Some of these cells are completely replaced by fat. Between 100 and 200 days transplants may reach the size of 3 or 4 mm. in diameter. The larger transplants may show adenomatous nodules at the periphery in which the typical cortical arrangement of the cells is seen. The oldest transplant we studied was 276 days, and it showed no signs of degeneration or exhaustion atrophy.

We studied homotransplants in 5 animals and found that during the first 2 weeks the changes are the same as in autoplastic transplants. During the 3rd week an occasional clump of regenerated suprarenal cells is seen, but foreign body reactions with giant cells and lymphocytes are already in evidence. By the 7th week most of the homotransplants are completely absorbed.

Functioning of the Transplants.

From these experiments we obtained evidence that autoplastic transplants are capable of maintaining the life of guinea pigs in the absence of both main glands and suprarenal accessories. If the transplants are small or are undergoing absorption, they maintain the animal in a poor state, which may continue for weeks until the guinea pig dies of suprarenal insufficiency. In those instances where the transplants reach full development, guinea pigs are maintained normally for a sufficiently long time to warrant the assumption that they can live their normal life period. We have observed 1 transplanted ·guinea pig surviving suprarenalectomy in good condition for 9 months.

Of 17 doubly suprarenalectomized and transplanted animals 5 died from suprarenal insufficiency within 5 days after removal of the second gland. 1 death occurred on the 1st day, 1 on the 2nd, 2 on the 4th, and 1 on the 5th. The autopsies showed complete removal of both

main glands, and no macroscopic accessories. Transplants had been inserted from 24 to 59 days previously, and had been absorbed in all but 1 animal which showed some clumps of suprarenal cells in each of several transplants. They were apparently insufficient to maintain life. 1 guinea pig died from a transverse myelitis due to osteomyelitis of the thoracic spine on the 7th day after removal of the second gland; another died of a general infection following an abortion on the 15th day; a 3rd died from pneumonia on the 24th day. These 3 had been transplanted 29 to 59 days previously, and each had active regenerating transplants.

One guinea pig was sacrificed in good condition 13 days after removal of the left gland and 57 days after insertion of the transplants. The autopsy showed complete bilateral ablation of the suprarenals, no accessories, and numerous pin-head-sized positive transplant areas which when sectioned showed groups of vascularized cortical cells.

Following the removal of the second gland, 4 guinea pigs died of chronic suprarenal insufficiency, 1 each on the 41st, 46th, 52nd, and 57th day. The transplants had been inserted 85 to 104 days previously, and on histological examination were degenerated and fibrotic. No accessories were found in these animals.

One animal killed 220 days after the removal of the second gland showed a large cortical accessory. No transplants were present. The remaining 3 animals were killed, 2 on the 124th, and 1 on the 221st day after the removal of the second gland. Each had several large vascular transplants which were apparently physiologically active, maintaining the animals in good condition. 1 of these animals had a 3 mm. accessory.

In summary we can present 11 guinea pigs upon which we base our conclusions regarding the functioning of the transplants. These animals after suprarenalectomy and transplantation either died of suprarenal insufficiency or were killed to terminate the experiment months after transplantation. At autopsy no accessory cortical tissue was found in any of these animals. Of the 11 animals, 6 lived longer than 40 days after the removal of the second gland. These figures stand in marked contrast to the average survival time given in the literature for double suprarenalectomized guinea pigs, and which we ourselves have found, that is, 3 or 4 days. They indicate that

transplants are capable of prolonging the life of suprarenalectomized guinea pigs or of maintaining them indefinitely.

DISCUSSION.

Irritation phenomena occurred in our experiments even when suprarenal cortex alone was transplanted, but were different in their appearance, severity, and consequence from those described by Elliott and Tuckett who transplanted both cortex and medulla. The cortical fragments were definitely palpable as firm plaques for about 3 days after their insertion due to the absorption of water by the lipoid-rich tissue. By the 4th or 5th day serosanguineous fluid had collected underneath the abdominal wound stitches of nearly all the animals. Sometimes 10 cc. of a bloody fluid could be expressed. Frequently on the 6th or 7th day the wound opened spontaneously, the skin edges sloughed out, and the transplants were destroyed. The edema and inflammatory reactions around the remaining transplants were followed by connective tissue growth, which impaired the vitality of the transplant during its organization through pressure and interference with the circulation. The result is frequently absorption of the transplant, but even if the transplant is not absorbed its growth is usually interfered with.

The reactions that occur with suprarenal transplantation in the guinea pig have not been conclusively explained. Elliott and Tuckett believed that the edema and solution of the guinea pig tissues were caused by some substance in the medulla, not epinephrine, and not present in all mammalian suprarenals. They reported that the irritant substance was diffusible and destroyed by heating to 65°C.

They excluded epinephrine as the toxic agent because the subcutaneous injection of 1 mg. in 1 cc. of salt solution caused a skin slough, but never the inflammatory reaction with edema and corrosion of the tissues such as is produced by the medulla. Even in lethal doses epinephrine did not cause an inflammatory reaction. It was further shown that this reaction did not follow the grafting of the whole gland on the peritoneum, or between the muscle and peritoneum. Neither do irritation phenomena follow the transplantation of the guinea pig suprarenal beneath the skin of the rabbit, rat, or cat. It

occurs only when the whole suprarenal is transplanted, auto-, homo-. or heteroplastically beneath the skin of a guinea pig. The appearance of these changes is peculiar to the suprarenal, for transplantation of the kidneys, spleen, thyroid, thymus, bone, and cartilage into the subcutaneous tissues of the guinea pig is not followed by edema or corrosion. Nor does transplantation either auto- or homoplastic of the suprarenal of the dog, cat, rabbit, or rat produce this phenomenon.

We believe that the milder reactions we observed following the transplantation of suprarenal cortex and the acute reactions described by Elliott and Tuckett are identical and are induced by the same irritating substance which however is present in greater concentration in the medulla. We believe that this substance has entered the cortex from the medulla through manipulation of the gland during its removal. We cannot agree with Elliott and Tuckett that the substance is neither epinephrine nor related to it, for the injection of epinephrine causes sloughing of the tissues. The difference between the reaction which follows the introduction of epinephrine alone, and that following the grafting of the entire suprarenal may be one of degree, due to the poulticing effect exercised by the necrotic transplant. Marine and Sandberg in some unpublished work concluded that epinephrine was the substance responsible for the phenomenon reported by Elliott and Tuckett. Some peculiarity of the guinea pig's subcutaneous tissues and abdominal muscles may in part be accountable for this idiosyncracy.

In our experiments the right gland was first removed and transplanted, and some weeks later the left gland was removed. This is the satisfactory way to study transplantation in an animal so susceptible to double suprarenalectomy. We cannot say whether a greater number of transplants would have taken and grown had a suprarenal insufficiency been induced by the removal of more than one gland at the initial operation, but it seems that the take of the suprarenal transplant is not necessarily dependent upon an insufficiency. 3 weeks elapse before the transplants regenerate, and at about this time the second gland is removed in most cases. This must be a stimulus for the growth of the transplants. Some respond and grow, while others degenerate even when so stimulated.

CONCLUSIONS.

1. The suprarenal cortex of the guinea pig can be transplanted autoplastically into the abdominal wall and may remain for months, growing to fairly large size.

2. The suprarenal cortex of the guinea pig can be transplanted homoplastically but these transplants usually degenerate after a few months.

3. Small autoplastic transplants are capable of maintaining the life of a completely suprarenalectomized guinea pig for weeks and large transplants maintain the animal indefinitely in good condition.

4. Irritation phenomena follow the grafting of suprarenal cortex due to the entrance of epinephrine into this tissue during the manipulation involved in removal of the gland.

EXPLANATION OF PLATE 19.

FIG. 1. Low power of a 59 day autotransplant. Regeneration is progressing actively though there is considerable scarring in the center of the graft.

FIG. 2. High power of Fig. 1 showing cortical cells in glomerular formation.

FIG. 3. Large completely regenerated suprarenal transplant 142 days after insertion.

FIG. 4. High power of Fig. 3 showing closely packed, highly vacuolated cortical cells.
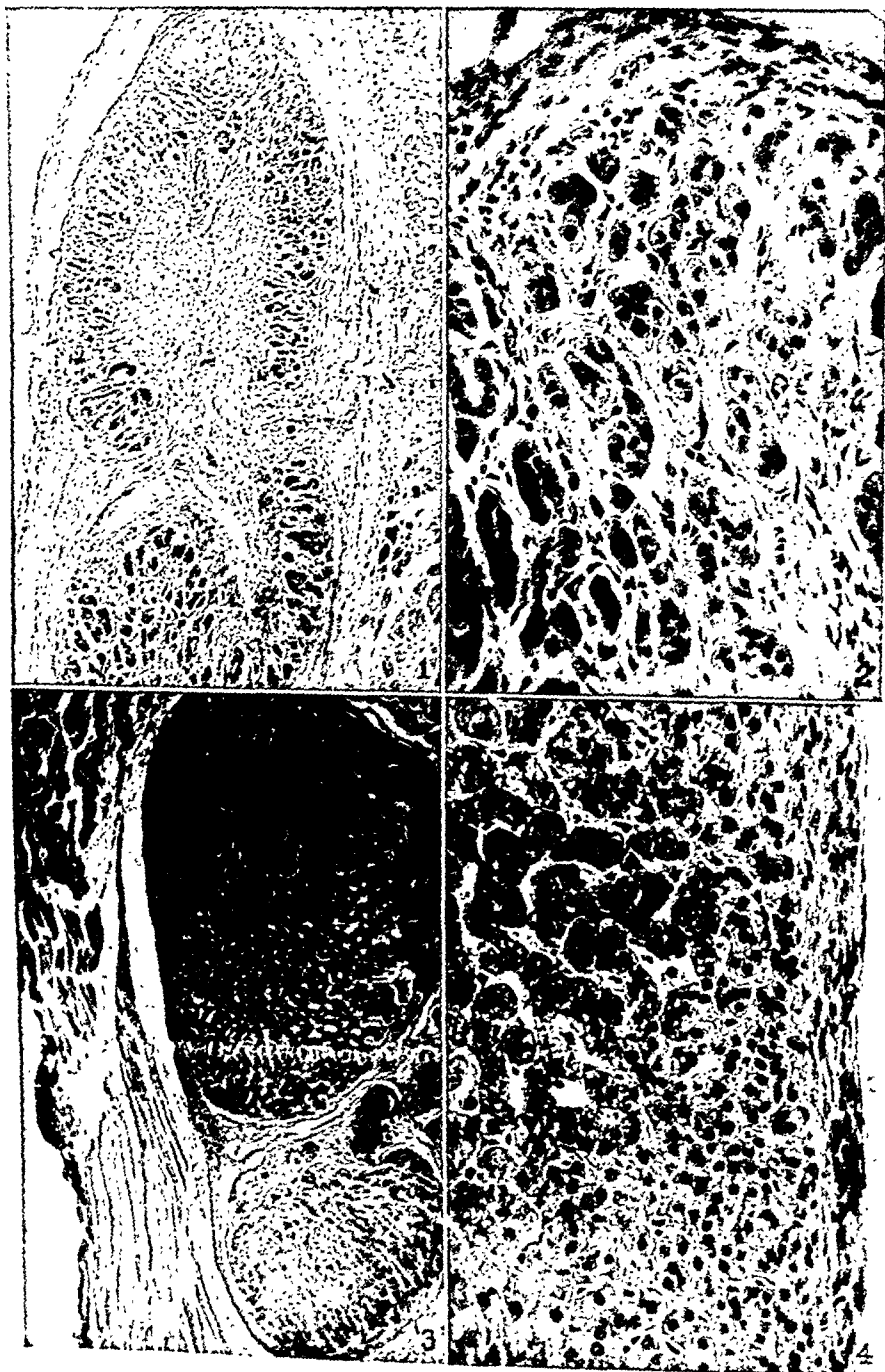
THE INFLUENCE OF THYROIDECTOMY, SPLENECTOMY, GONADECTOMY, AND SUPRARENALECTOMY UPON THE DEVELOPMENT OF EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS.*

BY SHEPARD SHAPIRO, M.D.

(From the Laboratory Division, Montefiore Hospital, New York.)

Ignatowski (1) showed in 1908 that in rabbits the prolonged feeding of foods of animal origin caused atherosclerosis of the aorta. He believed that the proteins were responsible for the intimal changes. It was subsequently found by Stuckey (2) and by Wesselkin (3) that only cholesterol-containing substances produced these arterial changes and it was finally determined that cholesterol was the essential constituent when Anitschkow and Chalatow (4) and Wacker and Hueck (5) produced atheromata of the aorta by feeding pure cholesterol in oil to rabbits. Later Aschoff and his coworkers (6) demonstrated that the anisotropic crystals deposited within the areas of intimal swelling and softening were cholesterol esters.

The similarity of experimental atheromata to those seen in humans was pointed out by various authors, especially Anitschkow (7), Aschoff (8), Zinserling (9), Klotz (10), and Bailey (11). It was recognized, as has been emphasized by Bailey (11), that although the experimental atheromata are practically identical with those seen in man, constantly associated with the artificially produced lesions are fatty deposits in other tissues such as spleen, liver, and kidneys not usually accompanying atherosclerosis in man. Anitschkow (12) therefore fed to rabbits over long periods of time (about 2 years) diets containing small doses of cholesterol, producing only very slight or no hypercholesterolemia. At autopsy these animals showed atheromata but no deposits in the other organs.

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* Presented in abstract before the American Society for Experimental Pathology, Cleveland, December 28, 1925.

Much additional work on experimental atherosclerosis has been done and numerous reports published from various sources, especially Russia, Germany, and this country. Historical reviews have been included in several of the older and more recent papers. Therefore, no attempt will be made at this time to cover this phase of the subject and those interested are referred to the publications of Rickett (13), Dewey (14), Schönheimer (15), Bailey (11), and Chuma (16).

Comprehensive descriptions of the intimal lesions as they occur in man and in rabbits following cholesterol feedings have been published by various authors, especially those already mentioned, and are so well known that we shall merely refer to them briefly. The lesions observed by us were identical with those reported by these workers and may be described as follows:

Blocks were taken from the thoracic aorta, usually at the arch, and fixed in 10 per cent formalin. Celloidin sections were stained with hematoxylin and eosin, frozen sections with Sudan III and hematoxylin, and unstained sections, mounted in glycerol jelly, were examined with the polarizing microscope. In some instances paraffin sections were stained with Weigert's elastic tissue stain and counterstained with Van Gieson's stain.

Because this was a comparative study we classified the lesions into (1) early atheromatosis, (2) moderate, and (3) severe atheromatosis.

1. Early Atheromatosis.—These were the earliest changes observed. There were sparsely distributed swellings of the vessel wall which macroscopically appeared as punctate elevations on the intima. Microscopically they consisted of local thickenings of the intima in which were present large irregularly shaped cells occurring in one or several layers and containing a substance which stained with Sudan III, a small amount of which was anisotropic. The internal elastic membrane and the outer layers of the aortic wall showed no alterations at this stage.

2. Moderate Atheromatosis.—Macroscopically the lesions were more numerous and larger. Microscopically at this stage there was further thickening of the intima with an increase in the number of large irregular "foamy" cells. The internal elastic membrane was more prominent and showed some separation of its fibers. Within the media there was a small amount of fat-staining material which was anisotropic.

3. Severe Atheromatosis.—In severe atheromatosis the lesions extended over the greater part of the intimal surface of the aorta. Microscopically the intima was much more thickened and the large foamy cells were still more numerous. Fibroblasts were visible. The Sudan-staining anisotropic material was more abundant both in the intima and media. There was much more separation of the elastic fibers at this stage.

More recent work by Murata and Kataoka (17) and by Chuma (16) has shown that castration tends to facilitate the development of atheromata after lanolin (cholesterol) feeding. Murata (18) reported further that thyroid administration to these rabbits inhibits atheroma formation.

Because of their susceptibility to the development of atherosclerosis rabbits have been used in these experiments. It has been possible also to produce the same changes in guinea pigs (Anitschkow and Chalatow (4), Schönheimer (15)) and in omnivorous animals, especially after castration (Löwenthal (19)).

The possible occurrence of spontaneous atheromata in rabbits is of the greatest importance in this work. There are many factors which may be responsible for the development of the condition, notably, age of the animal, hygienic conditions, previous infections, and diet. The frequency of spontaneous atherosclerosis has, therefore, been observed and commented upon by various authors. The findings, however, have been in wide disagreement. For example, Miles (20) found 17 out of 49 rabbits to have spontaneous atheromata while Steinbiss (21) could find none in 500 rabbits examined. Accordingly van Leersum (22) in 1914 tabulated the findings of various authors up to that time. Of 1937 rabbits examined spontaneous lesions were found in 49 instances. In addition to these Ophüls (23) has reported none present in 50 rabbits while Weinberg (24) found up to 9 per cent of 692 rabbits to have spontaneous atheromata.

Therefore, it is of the greatest significance that all the rabbits used in the experiments reported in this paper were of known age, were born and reared in this laboratory, lived under practically identical conditions since birth, and were apparently healthy and vigorous when selected for the experiments. Furthermore, it is noteworthy that of more than 250 rabbits which came to autopsy in this laboratory, a number of them litter mates of the rabbits used in these experiments, and which were examined for atheromata, only 1 had spontaneous lesions in the aorta. This rabbit had long standing snuffles and showed extensive chronic pulmonary disease at autopsy.

Cholesterol has been administered in various forms for the production of hypercholesterolemia and atherosclerosis. It has been given in pure form by intravenous injection in sesame oil (Adler (25)),

emulsified with sodium oleate (Klotz (10)), intraperitoneally in aqueous emulsion (Dewey (14)), and by feeding cholesterol-containing substances such as egg yolk, brain substance, dried liver (Anitschkow (7), Stuckey (2), Steinbiss (21), Bailey (11), and others). Lanolin (adeps lanæ hyd.) first used by Kon (26), contains cholesterol in large quantities and has been found to be an excellent means for introducing this substance into the body by feeding.

In a previous series of experiments (unpublished) we used the aqueous emulsion described by Dewey (14) intravenously and intraperitoneally but with unsatisfactory results. In the experiments herein reported we fed the lanolin in oil in addition to the regular laboratory diet of alfalfa hay, oats, and vegetables. The dose of lanolin used by us was 4 gm. daily in 12 cc. cottonseed oil. The mixture was heated to about body temperature and fed by pipette.

As has already been pointed out it has been found that cholesterol plays an essential rôle in the formation of experimental atheromata of the aorta. We have, therefore, studied some of the factors which are believed to influence the metabolism of this fatty substance and upon which might depend its abnormal deposition within the intima of the aorta. It is the purpose of this paper to present evidence to show the influence of feeding lanolin in oil on the production of experimental atherosclerosis in (1) normal rabbits, (2) thyroidectomized rabbits, (3) gonadectomized rabbits, (4) splenectomized rabbits, and (5) suprarenalectomized rabbits.[1]

In this report are included only the 44 animals which lived over 25 days. We excluded from the series 10 rabbits which died of marasmus due to toxicity of cottonseed oil and 4 which died of acute suprarenal insufficiency.

1. Normal Rabbits.—We found that rabbits with all organs intact sacrificed after 110 days of feeding with lanolin in oil showed early atheromata in the aorta. 3 rabbits sacrificed after 90, 95, and 100 days of the feeding respectively showed no atheromata. The others, killed after 110 days or longer, all showed atheromata.

2. Gonadectomized Rabbits.—Confirming Kon, Murata, and Chuma, we observed that gonadectomized rabbits developed atheromata more

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[1] All operations were performed under ether anesthesia.

readily than those with all organs intact. After 50 to 60 days of feeding with lanolin the castrates showed early atheromata. There were 2 exceptions: 1 sacrificed after 50 and the other after 90 days showed no atheromata.

TABLE I.

Number of rabbits in group	Operative procedure	Period of lanolin feeding	Degree of atheromatosis	Remarks
		days		
10	None	110–120	Early	
6	Gonadectomy	50–60	Early	Two fed 50 and 90 days showed no atheromata
7	Splenectomy	50–60	Early and moderate	
8	Thyroidectomy	40–50 70	Moderate Severe	
6	Double suprarenalectomy	85–100 110	None Early	Largest thyroids 8 to 10 times normal size. Lymphoid overgrowth
1	Thyroidectomy and double suprarenalectomy	50	None	
1	Thyroidectomy and splenectomy	50	Severe	
3 -	Double suprarenalectomy and splenectomy	45 106–110	None Early	
2	Gonadectomy and splenectomy	100	Severe	

3. Splenectomized Rabbits.—After 50 to 60 days of feeding with lanolin in oil the splenectomized rabbits showed early and moderate atherosclerosis. The lesions were more extensive than those seen in the castrated animals which were fed over equal periods of time.

4. Thyroidectomized Rabbits.—Thyroid-deficient rabbits showed

moderate atheromata after 40 to 50 days and severe atheromata after 70 days of feeding with lanolin. This group was the most susceptible.

5. *Suprarenalectomized Rabbits.*—10 doubly suprarenalectomized rabbits were used. Of these 1 was also thyroidectomized and 3 splenectomized. Of the first 6, 5 were fed between 85 and 100 days. None of these showed atheromata. The remaining one was sacrificed after 110 days of feeding. Early atheromata were present. The thyroidectomized and suprarenalectomized rabbit was fed 50 days. The aorta was free from atheromata at autopsy. Of the 3 splenectomized and suprarenalectomized, 1 fed 45 days on lanolin showed early atheromata.

At the same time, for controls, 1 thyroidectomized and splenectomized rabbit was fed 50 days and 2 gonadectomized and splenectomized rabbits were fed 100 days. At autopsy all 3 showed severe atheromata of the aorta.

<div align="center">DISCUSSION.</div>

We find, as have others, that deposition of cholesterol or cholesterol esters within the intima of the aorta initiates the cellular alterations which result in the formation of atheromata. We have readily produced it experimentally in rabbits by inducing prolonged alimentary hypercholesterolemia. We find further that thyroidectomy, splenectomy, and gonadectomy each facilitate and accelerate the development of this condition within the aorta. The thyroid-deficient rabbits are most susceptible and the splenectomized group are slightly more susceptible than the castrates.

The question arises: Why does removal of the gonads, the spleen, or the thyroid facilitate the development of experimental atheromata? In the case of the spleen we may be eliminating a possible storehouse for excess cholesterol because it sometimes undergoes very marked enlargement in the presence of prolonged lipemia. This is well illustrated in experimental lipemia and clinically in Gaucher's disease and in some cases of diabetic lipemia (1, 27, 5, 15, 28, 29, 30, and others). The relation of the spleen to the reticulo-endothelial system and its function in both the destruction of old red blood cells and the formation of new erythrocytes emphasizes the possibility that the spleen not only stores cholesterol but also utilizes it. Thus also it has been

found that splenectomy alone is followed by hypercholesterolemia (31–39).

Concerning the relation of the gonads to cholesterol metabolism very little is known. Their removal, however, may give rise to a slight hypercholesterolemia (40, 41). Certainly they do not store the fatty substance in any appreciable amount although the interstitial cells may appear more prominent in prolonged hypercholesterolemia. To what extent cholesterol is utilized in ovarian or testicular function still remains to be demonstrated. Clinically, menopause cases are said to show a tendency to develop hypercholesterolemia (42). One other possibility deserves emphasis, however, namely, the interrelation of the sex glands with the thyroid. It appears that a synergistic functional relationship operates between the gonads and the thyroid. For example, gonadectomy may cause a slight drop in heat production and even involution of the thyroid (43–49). Also, it is known that at those periods when the sex glands are the seat of both physiological and morphological alterations (puberty, pregnancy, climacteric) the thyroid may likewise undergo striking changes, sometimes to pathological extents so that exophthalmic goiter, simple goiter, or myxedema (Gull's disease) may result. In all probability, therefore, the importance of the gonads in cholesterol metabolism is dependent upon the interrelationship which exists between the sex glands and the thyroid (46, 50).

The thyroid on the other hand plays what appears to be an essential rôle in the metabolism of the fats and fat-like substances, including cholesterol. Exactly what processes in the burning of fats are dependent upon the presence of thyroxin for their completion cannot be stated at the present time. There is good evidence, however, that high fat diets cause increased thyroid activity. Indeed, unless iodine is administered to prevent it, thyroid overgrowth soon results. This has been amply illustrated in the work of Marine and Lenhart (51), McCarrison (52), the Mellanbys (53), and ourselves (54). We have observed further that such thyroid insufficiency produced by feeding neutral fat (cottonseed oil) over long periods of time is accompanied (after 60 days or longer) by hypercholesterolemia and that this increase in the cholesterol content of the blood is directly proportional to the degree of thyroid insufficiency (*i.e.* hyperplasia) (55).

We find that thyroidectomy in rabbits facilitates and accelerates the deposition of cholesterol and the development of experimental atheromata. The reason appears to be that thyroid-deficient animals are less able to oxidize fats and fatty substances (cholesterol) than normals. Marked hypercholesterolemia, cholesterol deposition, and, in turn, consequent atheroma formation result. In direct accord with this is the old and well known clinical observation that thyroid-deficient animals and man are very prone to develop hypercholesterolemia and atheromatosis (56–61, and others).

At this juncture it should be pointed out that since iodine is indispensable for thyroid function the beneficial effects known to follow the prolonged administration of iodides clinically in atheromatosis and atherosclerosis may be dependent upon the influence that such iodine compounds have upon thyroid activity. There is some evidence that the cholesterol content of the blood can be lowered by the administration of thyroid or iodine (62–64). Marine has demonstrated clearly the effects of small (physiological?) doses of iodine upon thyroid hyperplasia. It seems, however, that large or massive doses of iodine may give rise to additional and important reactions. This is the subject of some investigations now being conducted.

Our evidence indicates that sublethal suprarenal insufficiency does not aid in the deposition of cholesterol and the development of experimental atheromata.

The suprarenalectomized rabbits which we used had recovered from the acute insufficiency following the removal of at least one and three-fourths glands and were well compensated during the subsequent experimental period. That they suffered from varying degrees of suprarenal insufficiency was indicated by the fact that they showed at autopsy some lymphoid overgrowth involving both thymus and lymph nodes and very large hyperplastic thyroids, in 3 instances 8 to 10 times the average normal size. Complete double suprarenal-ectomy was not done in all cases. A fragment of one gland was left behind purposely in 4 of these animals. Thus, we found that rabbits in a state of sublethal suprarenal insufficiency were not more susceptible to the development of atheromata of the aorta following the feeding of lanolin than rabbits with all organs intact. These findings confirm indirectly the observations of Baumann and Holly (65). In 4 in-

stances (1 thyroidectomized and 3 splenectomized) we obtained evidence which suggests that such a state of suprarenal insufficiency may exert an inhibitory influence upon the development of experimental atherosclerosis.

A propos of this it might be mentioned that atherosclerosis is rare in individuals with lymphatism, status thymicolymphaticus, and similar conditions characterized by lymphoid overgrowth. A constitutional anomaly of similar nature seems to result in animals in which a state of sublethal suprarenal insufficiency has been experimentally produced (66).

All this evidence indicates that conditions which are accompanied by long standing hypercholesterolemia cause or facilitate the deposition of cholesterol within the intima of the aorta. This, we find, as have numerous other workers, initiates the formation of experimental atheromata.

In these experiments with lanolin feeding there was no hypertension (67). The blood pressure readings were determined by the method described by Anderson (68). In other words, we find that experimental atheromatosis in rabbits may develop in the presence of normal blood pressure.

However, we do not maintain that prolonged hypertension when present may not alter the arterial wall so that atheromatosis results. The same applies to other mechanical influences such as forces of pulling and dragging, and of wear and tear, the effects of which are seen so frequently in man in later life (Aschoff (69)). Our findings indicate that the deposition of cholesterol within the intima of the aorta initiates the formation of atheromata and any condition (hypercholesterolemia, mechanical forces, etc.) which predisposes to such precipitation thereby predisposes to the development of atheromatosis. This is in agreement with the findings of Aschoff and of Wacker and Hueck.

SUMMARY.

Alimentary hypercholesterolemia acting over a sufficient period of time (in rabbits with all organs intact, 110 days or longer) causes deposition of cholesterol within the intima of the aorta. Deposition of cholesterol within the intima of the aorta initiates the formation of

experimental atheromata. Thyroidectomy, splenectomy, and gona-
dectomy augment hypercholesterolemia and thereby facilitate and
accelerate the development of experimental atheromata of the aorta
in rabbits. Sublethal suprarenal insufficiency does not increase the
susceptibility of rabbits to the development of such atheromata.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

III. FURTHER STUDIES ON THE NATURE OF THE PURPURA-PRODUCING PRINCIPLE.

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INTRODUCTION.

It will be recalled from a previous (1) communication that pneumococcus extract was found to produce hemorrhagic purpura in laboratory animals.[1]   The purpura-producing principle resists oxidation and heating to 100°C. for 10 minutes; it is filter-passing; it is present in the fraction of pneumococcus extract obtained by full saturation with $(NH_4)_2SO_4$ after the acetic acid-precipitable substances have been removed.   The principle is not associated with type specificity or virulence of the organism and is distinct from the endohemotoxin always present in reduced extracts of Pneumococcus (2).   The opinion was expressed at the time that the purpura material was probably a degradation product of pneumococci, since it is present only when cell disintegration accompanied by autolysis has taken place.

Subsequent study (3), established the fact that pneumococcus extract is thrombolytic both *in vivo* and *in vitro*, and that the development of purpura in mice following the injection of pneumococcus extract is associated with an excessive diminution in the number of blood platelets, the greatest decrease usually occurring within 24 hours. In addition the red blood cells also are greatly reduced numerically,

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[1] Since our preceding paper, we have seen a reference of Carnot (Carnot, P., *Compt. rend. Soc. biol.*, 1899, li, 927) in which he describes reactions in rabbits to a "pneumococcus toxin." His descriptions indicate the development of purpura in these animals.

but the rate of their disappearance is slower than that of the throm-
bocytes. Although heat inactivates both the thrombolysin and
hemolysin, it has no effect on the purpura-producing property of
pneumococcus extract. Adsorption of extract with blood platelets
reduces the activity of the thrombolysin, but does not influence
the purpura-producing activity in animals.

In the present communication, further studies are reported upon
the nature of the purpura-producing principle. Observations are
recorded on (1) the immunological reactions, (2) the biological signifi-
cance and (3) the further chemical fractionation of this substance in
pneumococcus extracts.

I. Immunological Reactions of the Purpura-Producing Principle.

(a) *Antigenicity of the Purpura Material.*—The purpura-producing
substance, fractionated as will be described later, was used as immuniz-
ing antigen in rabbits. It was noted that during immunization,
purpura lesions which accompanied the earlier injections, were not
induced by the succeeding inoculations of the material. After
several weeks of immunization, the sera were tested for neutralization
of purpura in white mice and for the presence of pneumococcus anti-
bodies. Not only did these sera not possess the power of neutralizing
the purpura substance, as is brought out below, but they contained no
antibodies for the intact cell or its derivatives. Thus, precipitins
were not demonstrated for the purified purpura principle; for the
soluble specific substance of the homologous type of Pneumococcus,
nor for either extract or protein of the cell. Similarly, it was not
possible to show agglutinins for either the "S," encapsulated cell, or
the "R," capsule-free cell; nor were the sera able to confer protection
upon mice against infection with homologous or heterologous types
of Pneumococcus. In this respect, the purpura principle differs
markedly from the hemotoxin, since the latter is antigenic (2, 4).

(b) *Active Resistance to the Purpura Principle.*—The rabbits em-
ployed in the foregoing experiment indicated that notwithstanding
the fact that the purpura substance is non-antigenic, an increased
resistance may be acquired against purpura. In order to study
more thoroughly whether resistance can be exhalted actively, mice
were treated with heat-killed suspensions of Pneumococcus, or with

the protein (5), autolysate (1) or extract (6) of the bacterial cells. All the substances are known to stimulate the formation of antibodies (7) and the last two contain the purpura principle. Accordingly, mice received five weekly injections of the particular antigen either subcutaneously or intraperitoneally. 7 to 10 days after the final injection, the mice were tested for increased resistance to purpura.

TABLE I.

Increased Resistance to Purpura Acquired by White Mice Following Repeated Injections of Pneumococcus Extract.

Date of injection	Amount of extract injected	Number of mice injected	Number of mice developing purpura
	cc.		
Apr. 26	0.1	10	10
May 3	0.1	10	10
May 10	0.2	10	8
May 17	0.2	10	2
May 24	0.4	10	0
June 1	0.5	10	0

TABLE II.

The Protective Action of Immune Sera against Purpura in White Mice.

Sera	Number of mice injected	Mice developing purpura	
		Number	Per cent
Controls (no serum).........................	10	7	70
Normal serum.............................	20	16	80
Antipurpura...............................	10	10	100
Antipneumococcus.........................	5	5	100
Anti-R....................................	15	7	46
Anti-extract..............................	25	13	52
Anti-autolysate...........................	25	21	84
Antiprotein...............................	20	14	70

It was found that repeated injections of pneumococcus vaccine or protein do not increase the tolerance of mice to purpura. Mice so treated developed purpura following the injection of the same amount of extract as was required for the production of purpura in normal animals. Immunization to pneumococcus extract or autolysate, on the other hand, definitely renders mice more resistant to pur-

pura. This fact has been demonstrated satisfactorily a number of times, and a protocol typical of the results (Table I) shows that ten mice were made refractory to purpura by repeated injections of increasing amounts of pneumococcus extracts. It is also of note that the purpura substance no longer causes a diminution in the number of blood platelets or red blood cells in mice whose tolerance to purpura has been raised.

(c) *Passive Protection against the Purpura Principle.*—Whether white mice can be passively protected against purpura, was studied by the use of a number of immune rabbit sera prepared by immunization with the purpura principle itself, with "S" and "R" strains of Pneumococcus and with cell extracts, autolysates and the nucleoprotein fraction of the organism. In determining the neutralizing action of the sera against purpura, mixtures of 0.5 cc. of serum and 0.1–0.2 cc. of extract were injected intraperitoneally either immediately on mixing or after incubation together for 1 hour at 37°C. The sera prepared by immunization with the purpura material did not prevent the occurrence of purpura in mice. In no instance, moreover, did any of the sera provide definite passive protection against purpura. The data in Table II show the variation in susceptibility of individual mice and consequently the difficulty of evaluating experiments of this kind. While in the present instance 70 per cent of the normal mice developed purpura, other observations have shown variations from 100 per cent (*cf.* Table I) to 30 per cent of normal mice reactive to a fixed dosage. However, it is sufficiently clear that the sera included in the study did not confer upon normal mice protection against purpura. The lower percentage of animals developing purpura when anti-extract and anti-R sera were utilized, is suggestive of a partial protection, but experience with the purpura substance makes more plausible the interpretation of variation in susceptibility.

The evidence obtained, therefore, is that the purified purpura principle is non-antigenic, in the sense that it produces no demonstrable antibodies. White mice injected repeatedly with substances containing the purpura material acquire an increased tolerance to purpura. On the other hand, the sera of rabbits immunized with the purpura principle, the intact cell or its derivatives confer upon white mice no passive protection against purpura.

II. Biological Nature of the Purpura-Producing Principle.

It was shown in a preceding paper (1) that the purpura substance was found only in those cell derivatives which represented degradation products of pneumococci. The evidence thus far indicates that the substance is not contained as such in the cell, but rather that the purpura principle is a product accompanying cell autolysis. The present experiments substantiate this point of view and confirm the autolytic origin of the purpura material.

Attempts to obtain the purpura substance by dissolution of the cell with bile were repeated, but again the bile solutes were found incapable of producing purpura. Pneumococci were dissolved also by means of sodium oleate, but the resulting solutions did not contain the purpura principle. Curiously enough, incubation of bile solutes to allow digestion by the autolytic enzymes present in the cell did not yield the purpura substance.

Extracts prepared by rapidly freezing and thawing heavy suspensions of live pneumococci are actively purpura-producing, and there is reason to believe that this activity is dependent upon the action of the enzymes of the cell. Since the enzymes of Pneumococcus are inactivated (8, 9) by heating, whereas the purpura principle is resistant to heat (1), it was possible to determine the rôle of the enzymes in the derivation of the purpura substance. Extracts were prepared by freezing and thawing pneumococci previously exposed to 100°C. for 5 minutes. The extracts prepared from heat-killed cells, however, were found to be incapable of producing purpura in white mice. This is further evidence that the purpura principle is not preexistent in the cell.

Avery and Cullen (8, 9) have shown that cell-free filtrates of Pneumococcus contain, among other enzymes, a potent bacteriolytic enzyme which is operative on a substrate of heat-killed pneumococci. It became possible therefore to analyze the relationships of autolytic digestion to the formation of the purpura principle. A solution of active pneumococcus enzymes prepared according to the method of Avery and Cullen was allowed to act on cells killed by heating for 5 minutes at 100°C. After digestion at 37°C., there was distinct lysis of the organisms and cell-free filtrates were obtained after 24, 48,

72 and 96 hours. The filtrates, when injected into white mice, were found to be actively purpura-producing. The enzyme-containing extract, when diluted to the same extent as the filtrates obtained from the digestion experiment, did not produce purpura.

A pneumococcus enzyme solution was inactivated by boiling. It was then allowed to act on heat-killed pneumococci as in the above experiment. No lysis was observed, however, and the filtrates obtained from these tests did not produce purpura. Similar tests conducted with active enzymes in the presence of bile showed that the purpura substance was not demonstrable under these conditions.

The evidence indicates definitely, therefore, that the purpura principle is not a preformed constituent of the cell, but that it is a product of autolysis.

Avery and Cullen (9) have shown that the enzymes of Pneumococus cause lysis not only of Pneumococcus itself, but also of *Streptococcus viridans*. It was desirable, therefore, to determine whether purpura-producing properties could be obtained from streptococci by digestion with pneumococcus enzymes. Heavy suspensions of *Streptococcus viridans*, killed by heating at 100°C. for 10 minutes, were subjected to the action of pneumococcus enzymes as described in the preceding experiment. Filtrates obtained from the resulting bacteriolysis of streptococcus by pneumococcus enzymes were not capable of producing purpura in white mice.

Purpura is rarely seen in white mice during the course of experimental infection with Pneumococcus. This is possibly due to the fact that the virulent organisms are tremendously invasive and cause death rapidly. Since degraded and avirulent "R" cells yield as potent purpura-producing extracts as their virulent antecedents (1), a study of the disintegration of pneumococci provided a means for determining the occurrence of purpura following the injection of live pneumococci *in vivo*. Small and large amounts of concentrated suspensions of live "R" cells were injected both subcutaneously and intraperitoneally in mice. The majority of the mice survived but in no instance was purpura observed.

In summary, then, it can be said that the purpura-producing principle of Pneumococcus does not exist preformed in the cell. It definitely accompanies autolysis *in vitro* and, under the conditions

stated, purpura was not observed in animals injected with large amounts of live pneumococci.

III. Chemical Nature of the Purpura-Producing Principle.

Previous studies indicated (1) that the purpura substance was precipitated by full saturation with $(NH_4)_2SO_4$ after the nucleoproteins had been previously removed from pneumococcus extract. It was not clear, however, whether the purpura material was albumin or not. In the present study, further fractionation has been accomplished and the purpura principle has been separated from the original precipitate obtained with $(NH_4)_2SO_4$.

In the chemical purification, aqueous extracts of Pneumococcus were employed. Normal acetic acid was added to the extract until no further precipitation occurred. The acid precipitate was removed by centrifugation and the supernatant was withdrawn. The supernatant was heated to maximum coagulation, and the coagulum was whirled down. The materials removed by acid and by heat, as well as the supernatant after removal of both proteins, were studied.

As was previously shown (1) the acetic acid precipitate or nucleoprotein was not purpura-producing. The heat-coagulable proteins were irregular in their action and at best only faintly reactive. Since they were not washed, it seems likely that they were not entirely free from the supernatant. The supernatant remaining after both the acid-precipitable and heat-coagulable proteins had been removed, usually produced marked purpura in mice. The indications are therefore that the purpura principle is not present either in the acid-precipitable or heat-coagulable proteins.

The active purpura-producing supernatant as such gave no qualitative color tests for protein. When it was concentrated to one-tenth volume however, several tests were obtained which aid in identification of the principle. With the biuret test, a pink to purple color was observed. The xanthoproteic test was negative, and Millon's reagent gave a yellow to brown precipitate. A precipitate was obtained with full saturation of $(NH_4)_2SO_4$; the precipitate first appeared at about 60–70 per cent saturation and it increased in intensity up to full saturation. Both picric acid and trichloracetic acid gave precipitates, but in both instances the precipitates vanished

on heating and returned on cooling.   Since the concentrated solution, moreover, gave no further coagulation on boiling, it appears that the purpura-producing principle of Pneumococcus is identified with a primary proteose.   It cannot be said whether it is actually a proteose or some closely associated substance.   The purpura material is present in such small amounts and some is lost during fractionation, so that it becomes a difficult procedure to effect a further separation.

DISCUSSION.

The experiments recorded in this communication disclose that the purpura-producing principle of Pneumococcus does not stimulate the formation of demonstrable antibodies.   The serum of animals immunized with either the purpura principle itself, with pneumococcus cells or with extract, autolysate or nucleoprotein of the bacteria does not afford white mice passive protection against purpura.   In contrast to this fact, however, white mice and rabbits acquire an increased tolerance to purpura following successive injections of materials containing the purpura substance.   It seems likely, therefore, that the "active immunity" in this instance is really an increased resistance to the toxic agent.   This is supported by two facts: (1) the purpura-producing principle is not antigenic, and (2) passive protection against purpura is not transferred to normal mice by the serum of resistant animals.

The experiments on the biological nature of the purpura principle furnish direct evidence that it accompanies autolysis and that it is most probably a degradation product.   It was not obtained as a preformed constituent of the cell, since, in the absence of enzyme action, cytolysis of live or dead cells did not yield the purpura substance. Dissolution of the live cell by bile or sodium oleate, neither of which inhibits the activity of the purpura principle when once formed, did not furnish the purpura material.   Moreover, the presence of bile has been observed to inhibit definitely the formation of the purpura substance.   In fact Jobling and Strouse have shown that a number of unsaturated fatty acids, including sodium oleate, actually do inhibit the action of proteases.   Since bile does contain unsaturated fatty acid, there is some support for the belief that bile inhibits the digestive processes which give rise to the purpura prin-

ciple. Freezing and thawing heat-killed organisms in the absence of active enzymes did not liberate the purpura principle. On the other hand, the digestion of dead cells by active enzymes of Pneumococcus gave filtrates which were actively purpura-producing. In other words, the purpura substance appears to be an autolytic derivative of Pneumococcus.

Further fractionation of the purpura material indicates that it is closely related to a primary proteose. It has been definitely separated from both the acid-precipitable and heat-coagulable proteins of pneumococcus extract, but a more exact identification cannot be stated at the present time. In general proteoses have been found to be non-antigenic (10) although the observations of some investigators notably those of Fink (11) indicate that proteoses may be antigenic. That proteoses, moreover, may be toxic has been demonstrated by a number of investigators (12).

CONCLUSIONS.

1. The purpura-producing principle of Pneumococcus is non-antigenic in the sense that it does not stimulate the formation of antibodies.

2. White mice acquire an increased resistance to purpura as a result of repeated injections of toxic doses of the purpura substance.

3. The serum of rabbits immunized with the purified purpura principle, with "S" and "R" strains of Pneumococcus or with cell extracts, autolysates or the nucleoprotein fraction of the organism does not confer upon white mice protection against purpura.

4. The purpura principle does not exist preformed in the cell, but is rather an autolytic derivative; since it is formed only when pneumococci undergo autolysis, and it is not found when the autolytic ferments are inactivated.

5. The purpura substance is associated with the proteose fraction of active pneumococcus extracts.

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THE RELATION OF THE RETICULO-ENDOTHELIAL SYSTEM TO THE FORMATION OF AMYLOID.

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PLATES 20 TO 22.

In a recent article (1) Domagk states that he succeeded in producing amyloid in the spleen and liver of normal mice within 10 minutes by intravenous injection of a large quantity of living cocci; furthermore, that he could produce amyloid within 2 minutes in the spleen and liver of a mouse which had several injections of dead cocci previous to the final intravenous injection of living cocci. He also described marked changes in the reticulo-endothelial cells, and finally drew the conclusion that amyloid appears at first in the neighborhood of these phagocytic cells. This substance which he thought to be amyloid did not react to any of the specific stains (gentian violet or iodine), but showed metachromasia when stained with cresyl violet or Giemsa.

The Influence of the Injection of Dead and Living Bacteria upon the Formation of Amyloid.

Since vital staining by the intravenous injection of Congo red has proved in many experiments to be a most delicate and reliable, as well as specific, method for demonstrating even the earliest traces of amyloid (2), experiments were undertaken in order to define the nature of the substance produced in Domagk's experiments.

Each of a series of 10 mice was injected with 0.2 cc. of dead streptococci in the gluteal region four times at intervals of 2 days. Following these injections 0.5 cc. of living streptococci was injected intravenously into each animal. In another series of 10 mice 0.5 cc. of living streptococci was injected intravenously without any previous injections of dead bacteria. Corresponding animals of each group were killed 15 minutes, 30 minutes, 1 hour, 2 hours, 24 hours, 2 days, 3 days and 7 days after injection. Each of these animals received 0.5–0.7 cc. of 1 per cent solution of Congo red intravenously shortly before death.

None of the 20 animals showed even the slightest trace of amyloid in any of the organs, although many of them showed marked lesions in spleen and liver corresponding to those described by Domagk. The changes in the reticulo-endothelial cells are very marked and consist of swelling and vacuolation. These cells contain bacteria for some time after the injections and later on become distended with red blood cells and cellular debris.

These experiments obviously shed no light upon the relation of the reticulo-endothelial cells to amyloid formation since there was no evidence that amyloid was formed. The idea, however, that reticulo-endothelial cells could in some way be responsible for the formation of amyloid is very tempting in many respects. It would at once explain many morphological pictures seen in cases of experimental amyloidosis, the interpretation of which appears to be rather forced so far.

The Relation of the Reticulo-Endothelial Cells to the Site of Formation of Amyloid.

It is surprising indeed to find the phagocytic reticulo-endothelial cells of spleen, liver, kidney, adrenal and intestines located exactly in these places where amyloid is constantly seen. After one or more intravenous injections of 0.2 cc. of India ink in a mouse, the reticulo-endothelial cells filled with ink particles are seen forming black rings in the tissue around all the follicles of the spleen, while there is comparatively little ink phagocytized in other parts of this organ (Figs. 1 and 2). In the liver one finds ink in cells, located beneath the endothelium of the periportal vessels, and sometimes in the endothelial cells themselves. The many phagocytic cells in the capillaries and the Kupffer cells are loaded with ink particles and often appear as dark, solid black spots, their nuclei being unrecognizable. There are only a few of these cells seen around the central vessels (Figs. 3 and 4). In the kidney the ink is found in cells of the capillaries of the glomeruli, and in the medulla between tubules (Figs. 5 and 6); in the adrenals in phagocytic cells of the capillaries between the columns of the cells in the cortex; in the intestines most of the ink is seen in the stroma of the villi, especially at their tips, and also in the capillaries at their bases (Figs. 7 and 8). All of these are the very places where amyloid is invariably and typically found when present.

Another point in favor of the theory that the reticulo-endothelial

cells may play an active part in the formation of amyloid is the solitary appearance of small, isolated patches of amyloid in all of the organs concerned. This phenomenon can, of course, only be observed in very early stages, and is even then rarely seen in the spleen, which fact is responsible for the idea that amyloid appears at once in masses around the follicles. This is not true, for here, as elsewhere, there are first formed small isolated droplets of amyloid which anastomose later and form the solid ring which is so typical of this organ (Fig. 9).

In the liver the earliest form of amyloid is found in the form of small solitary patches in the tissue surrounding the periportal vessels and in the intermediate zone of the lobules (Fig. 4). These isolated areas soon unite, thus forming a complete layer of amyloid around the vessels. At the same time solid, anastomosing strands of this material are found in the adjoining parts of the liver lobules. The tissue around the central vessels is much less affected but not completely exempt. Some of the patches of amyloid appear to be located in the lumen of the capillaries, often completely surrounded by endothelial cells; this fact is mentioned by many authors and all sorts of explanations, which on the whole are rather unsatisfactory, have been made for this remarkable occurrence which seems to contradict any present theory of the mechanism of amyloid formation.

In the glomeruli and in the tissue of the papillæ of the kidneys, between the columns of cortical cells in the adrenals, in the stroma of the villi of the intestine, as well as in the tissue at their bases, one sees the earliest stage of amyloid represented by small isolated patches which later on form anastomoses which imitate the whole framework (Figs. 6 and 8). The same principle governs the appearance of amyloid in all these organs.

The solitary appearance of small patches of amyloid in different parts of different organs suggests its formation on the very spot where it is located rather than precipitation of a substance circulating in the blood stream. The assumption that amyloid is formed in some way by activities of specific cells would not be contradictory of morphological facts. It cannot, however, be too strongly emphasized that only studies of the early stages of amyloidosis are of any value as far as its genesis is concerned. In this sense the study of

chronic, even advanced cases, would be of about the same value as those of scars in questions of tuberculosis or syphilis.

Delay in Appearance of Amyloid Following Attempt at Blockage of Reticulo-Endothelial System with India Ink.

In order to demonstrate possible corelations between reticulo-endothelial cells and amyloid, 15 of a series of 30 mice (Series 1-b, Table I) were injected intravenously with India ink, and amyloid was then produced by subcutaneous injections of 0.5 cc. of 5 per cent nutrose as used by Kuczynski (3). An injection of 0.2 cc. of India ink was given 1 day before the first injection of nutrose. If such a mouse is killed 1 day after the injection, practically all the reticulo-endothelial cells are found to be loaded with ink particles. Later on one often finds cells, which have to be called reticulo-endothelial cells as far as their morphology and position go, without any ink particles in their protoplasm. This is not surprising since many of the ink-containing cells die and are replaced by new cells, most of which certainly contain no ink. The majority of the reticulo-endothelial cells, however, still show ink particles 2 months after a single injection. In general one can assume that most of the cells containing ink were present at the time of the injection.

On the same day the other half of the series (Series 1-a, Table I) were injected with nutrose only, for control purposes. At certain intervals animals were simultaneously killed from both series. The organs of mice which died spontaneously were fixed in Zenker, formalin and alcohol, and sections of the various organs stained for amyloid with Congo red (4) and gentian violet. All the other animals were injected intravenously with 0.5–0.7 cc. of 1 per cent Congo red at least ½ hour before death in order to mark out the amyloid vitally. Their organs were fixed in saturated corrosive sublimate.

In the mice injected with nutrose alone (Series 1-a, Table I) amyloid was found after about 30 daily injections, while all the previously killed animals showed none. The corresponding animals (Series 1-b, Table I) injected with India ink 30 days previously did not have any amyloid. These results suggested that the appearance of amyloid was retarded in those animals whose reticulo-endothelial cells were loaded with ink, but upon repeating the experiment (Series 2-a and b, Table I) amyloid was found to appear at about the same time in the mice receiving India ink and nutrose, as in those receiving nutrose alone.

In the attempt to settle this question of the possibility of influencing the time of appearance of amyloid by blocking the reticulo-endothelial cells with India ink, four more similar series of animals were studied. Realizing that only one injection of ink was perhaps not enough to block the reticulo-endothelial system very extensively and that this fact might have been responsible for the indecisiveness of

the second experiment, those mice receiving ink of each of these four latter series were injected three to ten times during the course of the experiment.

TABLE I.

The Relation of Attempted Blockage of Reticulo-Endothelial System by Injection of India Ink to the Time of Appearance of Amyloid.

| Repeated subcutaneous injections of nutrose 0.5 cc., 5 per cent solution 6 times a wk. | | | | Intravenous injection of India ink before and during the experiment. Repeated subcutaneous injection of nutrose 0.5 cc., 5 per cent solution 6 times a wk. | | | | |
Series	Duration of experiment in days	Numbers of mice	Amyloid	Series	Duration of experiment in days	Numbers of mice	No. of intravenous injections of India ink	Amyloid
1-a	30	1–13	—	1-b	30	1–13	1	—
	31	14	+		31	14	1	—
	32	15*	—		36	15	1	—
2-a	13	1–14	—	2-b	38	1–13	1	—
	45	15	+		44	14	1	+
					52	15	1	+
3-a	36	1–13	—	3-b	35	1–12	1–2	—
	38	14	+		37	13	3	—
	42	15	+		41	14	3	—
					50	15	3	+
4-a	6	1–13	—	4-b	14	1–12	1	—
	22	14	—		28	13	2	—
	25	15	+		38	14	3	—
					47	15	5	+
5-a	34	1–13	—	5-b	30	1–13	1–4	—
	36	14	—		36	14	5	—
	42	15	+		42	15	5	—
6-a	18	1–11	—	6-b	18	1–13	1–3	—
	42	12	—		42	14	7	—
	47	13	+		53	15	10	—
	48	14	+					
	53	15	+					

* Received one intravenous injection of India ink 15 days before death.

In each of these four series amyloid appeared later in those animals whose reticulo-endothelial cells were filled with India ink than in the controls receiving nutrose alone (see Table I).

Morphological Relationship of Newly Formed Amyloid and Ink-Containing Reticulo-Endothelial Cells.

In studying the morphological relations of reticulo-endothelial cells and amyloid, especially in differentiating reticulo-endothelial cells present before the appearance of amyloid and those formed during and after its formation, only mice of Series 1 and 2 receiving one injection of ink, were used. The findings are constant but difficult to interpret. The clear-cut structure of the liver makes it easier to study the relationship between reticulo-endothelial cells and amyloid in this organ than in spleen and kidney; therefore, most of the observations were made in the liver. An average periportal field shows the following picture. A ring of amyloid is found between the endothelium of the vessels and the liver cells. Cells containing ink granules may be seen lining the lumen but are more often found outside the zone of amyloid, less frequently within. These cells appear to be intact and their nuclei are present. Embedded in the substance of the amyloid one occasionally sees isolated small particles of ink, having no longer any obvious relationship with living cells; but often there is no trace of ink in relatively large solid masses of amyloid (Fig. 10). In earlier stages the localized patches of amyloid around the periportal vessels are often seen to be completely surrounded by ink particles of various sizes, while there is no trace of ink in the amyloid itself (Fig. 11). In the midzonal areas of the lobules the relations between cells containing ink particles and patches of amyloid are manifold. The area of amyloid may be covered on one or the other side by cells containing ink (Fig. 12). These cells, in addition to other cells which do not contain ink, may also be found completely surrounding the amyloid (Fig. 13). Sometimes, but not frequently, cells or remains of cells with ink granules lie in the center of a small, solid area of amyloid, which is arranged in concentric layers and in turn is separated from the liver cells by ink-containing reticulo-endothelial cells (Fig. 12). Some of these ink granules in the periphery of an area of amyloid do not seem to be located any longer in the cells (Fig. 14). It appears as though these particles represent remains of cells which no longer exist. Not infrequently separate contiguous, crystalline patches of amyloid, completely

surrounded by liver cells, were found. Cells which contain ink granules separate these patches from the liver cells and there are also a few small particles of ink between them (Fig. 15).

In the spleen the cells which contain ink are widely scattered throughout the areas of amyloid, especially in the periphery of the zone of amyloid surrounding the follicles (Fig. 16). Isolated phagocytic cells are frequently found completely embedded in amyloid and free particles of ink are often enough seen lying in this substance (Fig. 17). Solitary patches of amyloid in the pulp are usually surrounded by cells which contain ink, sometimes only by ink granules (Fig. 18).

The interpretation of these findings in the liver and spleen appears difficult. One thing seems to be certain, that the reticulo-endothelial cells as such are not primarily transformed into amyloid. Were this true one would expect to find amyloid constantly containing large numbers of ink granules; this, however, is not the case. The granules which are actually seen included in masses of amyloid all appear to be the remains of cells passively involved in this process.

For pictures where the patches of amyloid are completely surrounded by cells containing ink, it is difficult to offer any other explanation except that these patches are situated in capillaries; otherwise how could amyloid, were its position outside the wall of capillaries, be surrounded by reticulo-endothelial cells which contain ink injected 6 weeks previously. Some of these cells may be phagocytic wandering cells, but most of them are certainly reticulo-endothelial cells as far as their morphology goes.

Only occasionally is there a suggestion that amyloid is situated within reticulo-endothelial cells. Fig. 19 shows two of these cells which seem to be filled with both ink and amyloid. Such appearances are very rarely seen.

Although one cannot always clearly interpret these various morphological relationships of amyloid, reticulo-endothelial cells and ink, none of them are contradictory to the idea that amyloid may in some way be formed through the activity of reticulo-endothelial cells. On the other hand, however, certain pictures (Figs. 13–15) demand this interpretation.

Injection of India Ink in Mice Already Showing Amyloidosis.

If one assumes that the reticulo-endothelial cells form amyloid, the question arises whether such an unusual process does not alter the cells greatly, or whether this substance is perhaps only formed by damaged and altered cells.

Should the reticulo-endothelial cells be involved only secondarily one would expect still to find many of these cells, which are yet phagocytic, in areas of already developed amyloid. There should also be ink-containing cells found in the periphery of follicles in the spleen and around the periportal fields of the liver after an injection of India ink in an animal having amyloidosis. One should expect to find their number decreased but there would be definite evidence of the presence of many of them which would take up the ink in a way similar to that of a normal animal. A mouse having received about 50 subcutaneous injections of nutrose was injected for the first time with 0.2 cc. of India ink intravenously 1 day before it was killed, and received $\frac{1}{2}$ cc. of 1 per cent Congo red $\frac{1}{2}$ hour before its death.

The sections of the organs of this animal show striking pictures. There are huge masses of vitally stained amyloid around the follicles in the spleen, and there are amyloidal rings around the periportal vessels of the liver, and some patches of amyloid in the intermediate zones.

There are almost no reticulo-endothelial cells containing ink granules around the follicles except in places where there is no amyloid, or where the mantle of amyloid around the follicles is not complete. Only a few of these cells are seen in large areas of amyloid (Fig. 20).

In the liver there are numerous cells loaded with ink far away from the periportal fields, while they are practically missing in a broad zone around the vessels themselves, where amyloid is most marked (Fig. 21). Identical pictures were seen in organs of mice injected three to five times subcutaneously with 1 cc. of a 1 per cent solution of trypan blue after the development of amyloid.

The striking absence of phagocytic cells in the areas where amyloid is most abundant must mean that the reticulo-endothelial cells of these areas have been destroyed or have been markedly altered physiologically. Were such pictures exceptional one might think that ink had

not reached the reticulo-endothelial cells due to obstruction of the periportal capillaries by amyloid and that ink reached only the reticulo-endothelial cells in the central portion of the lobule through collateral circulation from adjacent lobules. This conception of vascular obstruction is hardly tenable, however, for every capillary throughout the liver shows the usual amount of reticulo-endothelial cells containing ink particles. The additional fact that the amyloid throughout the entire lobule is brilliantly stained vitally by intravenous injections of Congo red also strongly speaks against a vascular obstruction in these areas.

Evidence of destruction of reticulo-endothelial cells is commonly found in cases of progressing amyloidosis in all the involved organs, and by the observations just described the idea of the direct connection of these perishing cells with the formation of amyloid is strongly supported, but there is no evidence that the cells as such are directly and primarily converted into amyloid.

DISCUSSION.

In this paper facts have been assembled which speak in favor of the theory that amyloid is actively formed by specific phagocytic cells (reticulo-endothelial cells) in the place where it appears. It must be said that all of the material collected does not furnish conclusive proof of this theory, but puts forward facts which suggest it rather strongly.

It is remarkable indeed to find most of the reticulo-endothelial cells in the organs in question in the very places where amyloid lies. The objection of having phagocytic cells in numbers in other organs also where amyloid is not commonly found, for instance in the lungs, must not necessarily overthrow this theory. Having phagocytic properties in common with those of the lungs, the reticulo-endothelial cells of spleen, liver, kidney, intestine, adrenal and lymph glands probably have many other special functions besides, each group adapting itself to the function of. the organ in which it is located. This special functional relationship may be the explanation why the reticulo-endothelial cells of spleen, liver and kidney should be more concerned than those of other organs in the formation of amyloid.

The solitary patchy appearance of young amyloid seems to indicate

its formation right on the spot where it appears rather than a precipitation of a substance circulating in the blood stream. That it appears in this form just at the very place where phagocytic cells are found in great number is also in favor of the theory that it might have been formed actively by specific cells. The presence of masses of amyloid in the lumen of capillaries of the liver, surrounded completely by Kupffer cells, could only be explained in this way.

The presence of two or more patches of amyloid lying independently but close together in the lumen of one capillary seems to indicate the mechanism by which continuous streaks of this material are formed. This is the form in which amyloid is seen in advanced cases.

It seems much easier to explain the endothelial covering of amyloid in chronic cases, which makes the amyloid appear to be outside the lumen, by a new growth of endothelial cells making an effort to preserve the smooth lining of the capillary than to interpret the presence of endothelial cells around the amyloid by a new growth of endothelium between amyloid and liver cells. The latter would mean a complete reorganization of the capillary system.

There are signs of severe damage and disintegration of reticulo-endothelial cells, during the process of formation of amyloid, as seen by direct observation and by the absence of cells which phagocytize India ink in the areas of newly formed amyloid. The presence of amyloid itself is a manifestation of such an unusual unphysiological condition that it is not difficult to believe that the material is formed by reticulo-endothelial cells, in an abnormal state.

The reticulo-endothelial cells are also secondarily involved in this process; they become partly and completely surrounded by amyloid and are often found embedded in this substance. Finally the cells die, leaving only their phagocytized granules in their places. It is impossible to say what becomes of their cell substances, whether they turn into amyloid or whether they are simply replaced by this substance.

The results of the experiments in which the attempt was made to block the reticulo-endothelial system with India ink have limited value for two reasons. In the first place it is impossible to block these cells completely; secondly there is a considerable irregularity in the time necessary for the experimental production of amyloid follow-

ing even standardized injections of nutrose. But since the attempted blockage of the reticulo-endothelial cells did result in marked delay of the appearance of amyloid, this fact, though itself inconclusive, at least supports the other observations leading to the idea that these cells are concerned in the formation of amyloid.

CONCLUSIONS.

The facts presented suggest strongly that reticulo-endothelial cells are actively concerned in the formation of amyloid. Points in favor of this theory are as follows:

1. The appearance of amyloid in places where reticulo-endothelial cells are normally present, sometimes in very large number.

2. The formation of early amyloid in the small solitary patches, which suggests its local formation.

3. The occurrence of solitary patches of amyloid apparently located within the capillaries of the liver.

4. The manifold relations between reticulo-endothelial cells marked out by phagocytized ink granules, loose ink particles and amyloid, described in the text.

5. The impossibility of demonstrating reticulo-endothelial cells in areas of forming amyloid by intravenous injections of India ink.

6. The delayed appearance of amyloid in animals after blockage of the reticulo-endothelial cells by repeated intravenous injections of India ink.

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EXPLANATION OF PLATES.

PLATE 20.

FIG. 1. Follicles of spleen. Reticulo-endothelial cells containing phagocytized ink particles around follicles. Three intravenous injections of 0.2 cc. of India ink. Unstained section. × about 75.

FIG. 2. Follicles of spleen. Vitally stained amyloid around follicles. Unstained section. × about 75.

FIG. 3. Peripheral vein of liver. Reticulo-endothelial cells with phagocytized ink particles beneath the epithelial lining and in capillaries. Ink granules in endothelium of vein. One intravenous injection of 0.2 cc. of India ink. Unstained section. × about 110.

FIG. 4. Peripheral vein of liver. Vitally stained solitary patches of amyloid in tissues around the vein and in the intermediate zone of liver lobule. Unstained section. × about 110.

FIG. 5. Cortex of kidney. Phagocytized ink particles in reticulo-endothelial cells of glomeruli. One intravenous injection of India ink. Unstained section. × about 110.

FIG. 6. Cortex of kidney. Vitally stained patches of amyloid in glomeruli. Unstained section. × about 110.

FIG. 7. Intestine. Reticulo-endothelial cells containing ink particles in tissues of villi. Three intravenous injections of India ink. Unstained section. × about 75.

FIG. 8. Intestine. Vitally stained amyloid in stroma of villi. Unstained section. × about 75.

FIG. 9. Spleen. Vitally stained patches of amyloid around follicles and in pulp of spleen. These patches are seen to become confluent in a semicircular area around the follicles. Unstained section. × about 75.

PLATE 21.

FIG. 10. Peripheral vein of liver in the center; the light zone around the endothelial lining represents amyloid. Patches of amyloid in the intermediate zone of lobule. Ink particles are found in endothelium, in reticulo-endothelial cells around the amyloidal ring, embedded in amyloid and in capillaries of the liver lobule. The solitary patches of amyloid are partly or completely surrounded by these cells. Fine ink granules in amyloidal zone around the vein. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14. Series 2-b.

FIG. 11. Part of the wall of a periportal vein of liver. Small area of amyloid (a) in wall of vein, surrounded by loose ink particles. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 15. Series 2-b.

Fig. 12. Intermediate zone of liver lobule. Solitary and confluent patches of amyloid. Reticulo-endothelial cells marked out by ink are found around these patches, lining them partially or completely. Ink-containing cells and fragments in the centers of two patches which show a concentrical arrangement (*a*). Fragments of cells and ink granules around small solitary patches of amyloid (*b*). Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14.   Series 2-b.

Fig. 13. Intermediate zone of liver lobule. Solitary patch of amyloid showing outspoken crystalline structure. Reticulo-endothelial cells with phagocytized ink particles surrounding the amyloid completely. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

Fig. 14. Intermediate zone of liver lobule. Solitary patch of amyloid showing outspoken crystalline structure. The amyloid is surrounded by reticulo-endothelial cells containing ink and by ink granules. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

Fig. 15. Intermediate zone of liver lobule. Three solitary patches of amyloid all showing crystalline structure. *a* and *b* are contiguous, but not confluent. Reticulo-endothelial cells containing ink around *a* and *b*. Fine ink particles between *a* and *b*. Cells with and without phagocytized ink particles around *c*. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

PLATE 22.

Fig. 16. Follicle of spleen. *a*, central artery. Zone of amyloid around the follicle. Reticulo-endothelial cells containing ink are scattered throughout the amyloid, but are more frequently seen in the periphery of the amyloidal zone and adjacent tissue. Isolated ink-containing cells and loose ink granules embedded in substance of amyloid. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 190. Mouse 14. Series 2-b.

Fig. 17. High power magnification of one part of Fig. 16. Malpighian body (central artery *a*) surrounded by amyloid (unstained). Isolated cells, some containing phagocytized ink particles embedded in amyloid. Also fine ink granules in substance of amyloid. × about 345.

Fig. 18. Splenic pulp. The large light areas are solitary patches of amyloid in tissue of pulp surrounded by reticulo-endothelial cells containing ink and also by ink granules. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 15. Series 2-b.

Fig. 19. Intermediate zone of liver lobule. Two small solitary patches of amyloid; *a*, almost surrounded by a solid black zone of ink; *b*, enclosed by ink particles which show a bipolar arrangement. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14. Series 2-b.

FIG. 20. Follicles of spleen partially surrounded by large masses of vitally stained amyloid. 0.2 cc. of India ink was injected 1 day before death. No reticulo-endothelial cells containing ink around follicles except in area *a* where the amyloidal zone around the follicle is not complete. (Compare with Fig. 1.) Unstained section. × about 75.

FIG. 21. Two periportal vessels of liver. Vitally stained rings of amyloid around vessels, patches of amyloid in intermediate zone. 0.2 cc. of India ink was injected 1 day before death. No reticulo-endothelial cells in tissue around both vessels, no ink granules in endothelium. The capillaries show the usual amount of phagocytic cells loaded with ink. (Compare with Fig. 3.) Unstained section. × about 115.

PLATE 20.

(Smetana: Reticulo-endothelial cells and amyloid.)

PLATE 21.
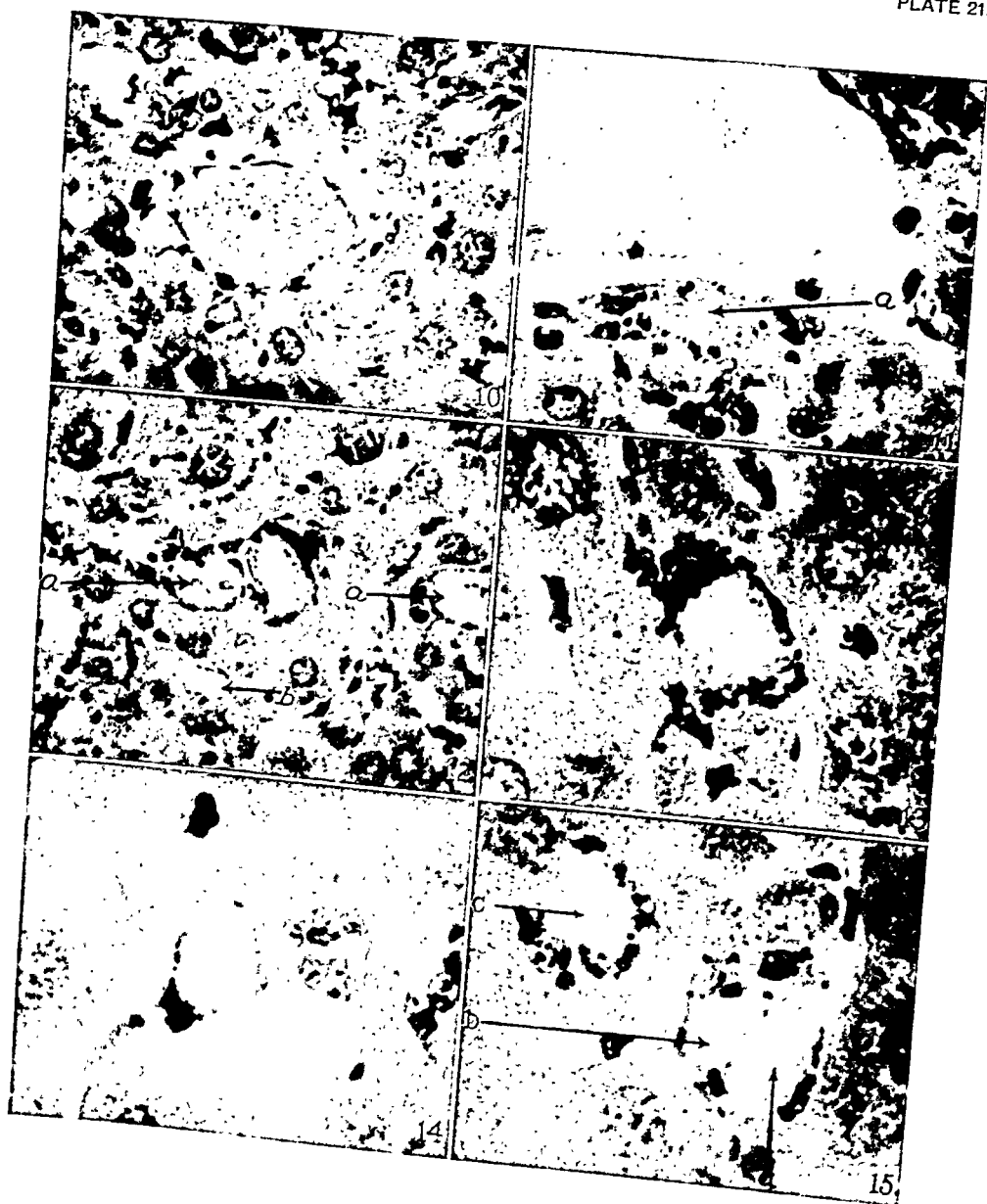
(Smetana: Reticulo-endothelial cells and amyloid.)

CHANGES IN CARBON DIOXIDE TENSION AND HYDROGEN ION CONCENTRATION OF THE BLOOD FOLLOWING MULTIPLE PULMONARY EMBOLISM.

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INTRODUCTION.

In an effort to explain the cause of the extraordinarily accelerated respirations which result from multiple experimental embolism of the arterioles and capillaries of the lungs (1) it seemed important to observe what changes occurred in the blood in respect to its carbon dioxide tension and hydrogen ion concentration. The relative importance of these two factors in the control of respiration has been much discussed in the literature of late years, and opinion has emphasized first one, then the other. It is probable that both the pressure of carbon dioxide and the hydrogen ion concentration of the blood (or of the respiratory center itself) play an important rôle in the chemical regulation of respiration. The characteristic breathing of acidosis, due to the retention of non-volatile acids, such as is seen in diabetic coma, is hyperpnea, or a deep, labored, slow type of breathing. This, too, is the character of respiration which results from inhalation of gas mixtures rich in carbon dioxide (5 to 10 per cent). As far as we know, rapid and shallow breathing has not been definitely related to a retention of carbon dioxide, nor to an increase in the concentration of hydrogen ions. In at least one clinical condition (bronchopneumonia) (2), however, an increased content of carbon dioxide in the blood has been described, and we know that in this condition the respiratory rate may be elevated. That the accelerated respirations which we were attempting to explain resulted from an increased carbon dioxide tension or a fall in pH seemed unlikely from the outset. Still we considered it necessary to make actual measurements of these changes

in relation to the changes in respirations before ruling them out as of importance. Especially was this true in view of the observation made by Dunn (3) that the inhalation of 10 per cent carbon dioxide by goats, breathing rapidly as the result of multiple pulmonary emboli, caused a reduction of the respiratory rate. In the light of our own observations, it seems probable that the cause of this reduction was probably an increased depth of respiration stimulated by the carbon dioxide in the inspired air. Such an increased depth would lead to an increase in alveolar oxygen tension and hence a decrease in the anoxemia. Anoxemia has been shown (1) to be a contributing factor to the rapid and shallow breathing resulting from multiple embolism of the pulmonary arterioles and capillaries, though not its cause.

EXPERIMENTAL.

The experimental procedure was a simple one. Dogs were anesthetized by the intravenous injection of barbital-sodium. A cannula was inserted into the left femoral artery, from which samples of blood were drawn for analysis, and into the right external jugular vein into which suspensions of potato starch cells were slowly injected. The blood was drawn without exposure to air and kept in sampling tubes over mercury. Analyses of carbon dioxide content were made in duplicate on the separated plasma or serum by the method of Van Slyke and Neill (4). The pH was determined on serum by the colorimetric method of Hastings and Sendroy (5). From these analytical data and from the following formula (6) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pk' to be 6.115:

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter. In some experiments the tidal air and minute volume of pulmonary ventilation were measured by securing a tightly fitting mask to the dog's muzzle and collecting the expired air by means of flutter valves in a Tissot spirometer. In other experiments the respiratory rate was simply counted by observing the animal's thoracic movements. In most of the experiments, anoxemia was prevented by permitting the animal to breathe oxygen. It was soon learned that the rapid and shallow breathing which comes on after a certain volume of starch suspension has been injected, is usually associated with an increase in the carbon dioxide tension of the plasma and an accompanying fall in pH. But subsequent observations revealed the fact that these changes do not necessarily occur, and sometimes follow rather than precede, the onset of abnormally accelerated respirations.

To test the effect of the experimental conditions *per se*, without starch injection, on the carbon dioxide tension and hydrogen ion concentration, these measurements were made on a dog at approximately hourly intervals over a period of 3 hours. The results showed a fall in pCO_2 and a rise in pH, or a change in the opposite direction from that usually encountered following embolism. These facts are shown in Table I.

In the following three experiments (Nos. 4, 7 and 9) the accelerated respirations following starch embolism were accompanied by a marked

TABLE I.

Experiment 6. Effect of Barbital-Sodium Anesthesia on pCO_2 and pH of Dog's Blood and on Respiratory Rate.

Time	Respiratory rate per min.	CO₂ content	pCO_2	pH
		mм	*mm.Hg*	
2.47	38	23.37	46.20	7.30
4.00	34	22.02	38.20	7.36
5.00	36	21.67	36.80	7.37

TABLE II.

Experiment 4. Effect of Intravenous Starch Suspension Injection on Respiratory Rate, CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	CO₂ content	pCO_2	pH
			mм	*mm.Hg*	
4.04		16	21.17	47.50	7.23
4.12–4.32	Injection of starch suspension				
4.33		52			
4.35		51	22.28	60.68	7.15

increase in CO_2 tension (from 5 to 13 mm.) and a definite fall in pH (from 0.07 to 0.09). In each instance characteristic rapid and shallow breathing occurred, the rate increasing from an average of 18 to the minute to an average of 46, and the depth decreasing accordingly. The experimental data are brought out in Tables II to IV. It is to be noted that the actual change in CO_2 content expressed in millimols may be slight when the change in CO_2 tension is considerable.

Examination of Table III will bring out the following facts: In a

TABLE III.

Experiment 7. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH	Arterial blood		
								O₂ content	O₂ capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
12.12	Dog breathing room air	17	174	2.96	23.60	37.56	7.40	7.46	8.70	85.8
12.22	" "									
12.45–12.51	Injection of starch suspension									
12.53	Room air	46			25.10	40.82	7.39	7.26	8.96	81.1
1.00	" "	50	130	6.48	23.25	44.00	7.32	6.81	8.80	77.4
1.13	Dog breathing 95 per cent O₂									
1.20		50	123	6.18						
1.26					23.52	45.52	7.31	8.87	9.36	94.8

TABLE IV.

Experiment 9. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH	Arterial blood		
								O₂ content	O₂ capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
11.59	Breathing 95 per cent O₂ throughout experiment									
12.05		21	150	3.16	23.10	34.36	7.43			
12.17–12.26	Intravenous injection of starch suspension									
12.29		37	124	4.62	22.75	39.46	7.36	9.00	8.42	107

dog anesthetized with barbital-sodium, the intravenous injection of a suspension of potato starch grains resulted in an increase in respiratory rate from 17 to 50 per minute. This was associated with a decrease in tidal air amounting to 44 cc., or a 25 per cent decrease, and a resulting increase in the volume of pulmonary ventilation from 2.96 liters to 6.48 liters per minute. This change occurred while the animal was breathing room air and was accompanied by a drop in the oxygen saturation of the arterial blood from 85.8 per cent to 77.4 per cent. Accompanying this the $p\mathrm{CO}_2$ rose from 37.56 mm. to 44.00 mm., with practically no change, however, in the CO_2 content of the serum as expressed in millimols. The hydrogen ion concentration, however, increased, as shown by the drop in pH from 7.40 to 7.32.

At this point oxygen was administered to the animal, with the result that the arterial oxygen saturation rose to 94.8 per cent, or the normal level, without, however, affecting the rate or depth of breathing, which still remained rapid and became even shallower than it had been. Nor was there a return of the $p\mathrm{CO}_2$ or pH to the former level.

From these experiments it can be concluded that embolism due to intravenous starch suspension leads to rapid and shallow breathing, which is accompanied by a fall in the percentage oxygen saturation of the arterial blood, a rise in the $p\mathrm{CO}_2$ and a fall in pH.

Restoring the blood to its normal oxygen saturation affects neither the respiratory rate or depth nor the carbon dioxide tension or hydrogen ion concentration. Similar results, as far as changes in $p\mathrm{CO}_2$ and pH are concerned, were observed in Experiment 9, in which the animal was permitted to breathe oxygen throughout, so that the arterial blood remained completely saturated with oxygen. In this experiment the respiratory rate rose from 21 to 37 following starch injection, the tidal air falling from 150 cc. to 124 cc. A slight drop in the millimolecular concentration of CO_2 occurred, from 23.10 mm to 22.75 mm, but with a fall in pH from 7.43 to 7.36, the $p\mathrm{CO}_2$ may be calculated to have risen by slightly more than 5 mm. These changes are brought out in Table IV.

Thus far no conclusions can be drawn as to whether the observed changes in $p\mathrm{CO}_2$ and pH are the cause of the accelerated respirations or whether they result from the same condition which gives rise to rapid and shallow breathing.

To observe the effect on respiration of increasing the partial pressure of CO_2 in the serum without the disturbing influence of pathological changes in the lungs, such an increase was produced by permitting a dog to inhale from a Douglas bag containing a 10 per cent CO_2-90 per cent oxygen mixture. In this experiment (No. 12) in which the CO_2 tension of the serum was raised by CO_2 inhalation there was naturally an increase in the hydrogen ion concentration, both changes being consonant with those observed in the starch experiments. Instead of the development of rapid and shallow

TABLE V.

Experiment 12. Effect of Inhalation of 10 Per Cent CO_2-90 Per Cent O_2 Mixture on pCO_2, pH of the Blood and on Pulmonary Ventilation.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH
			cc.	*liters*	*mM*	*mm.Hg*	
12.29	Breathing 95 per cent O_2	25.0	170	4.26			
12.35	" 95 " " "				26.21	40.76	7.41
12.38	" 95 " " "	30.4	194	4.90			
1.56	" 95 " " "	21.4	175	3.74			
2.03	" 95 " " "				26.28	42.82	7.39
2.05	" 95 " " "	21.4	180	3.86			
2.13–2.42	Breathing {10 per cent CO_2 {90 " " O_2						
2.30		42.0	390	16.42			
2.40					30.63	67.45	7.24
2.42		42.4	383	16.24			

breathing, however, the respirations, though they accelerated, more than doubled in depth. The average rate before CO_2 inhalation was 24, with a tidal air of 180 cc., compared to a rate of 42 after, with a tidal air of 383 cc. This was associated with a rise of CO_2 tension of nearly 25 mm., and a drop in pH from 7.40 to 7.24. These changes are exhibited in Table V.

This experiment (No. 12) is included simply for the purpose of demonstrating the well known effect of increasing the CO_2 tension and the hydrogen ion concentration, on the respirations. The significant difference from the starch effect is the marked increase in

depth. In many instances breathing high concentrations of CO_2 will increase the depth of respirations with little effect on rate.

Since, from the foregoing three experiments (Nos. 4, 7 and 9) it was impossible to say whether the changes in pCO_2 and pH antedated the onset of accelerated respirations, it was planned to draw the blood for analysis immediately after acceleration had begun. By this maneuver it was hoped to determine which change occurred first, *i.e.*, the change in CO_2 tension and pH or the change in rate and depth

TABLE VI.

Experiment 14. The Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth, and on CO_2 Tension and pH of the Blood.

Time.	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH
			cc.	*liters*	*mM*	*mm.Hg*	
11.43	Dog breathing 95 per cent O₂ throughout experiment	23.6	107	2.52			
11.49					24.40	51.48	7.27
11.52		23.2	120	2.78			
12.00–12.09	65 cc. 2 per cent starch suspension injected intravenously						
12.15		42.4	95	3.96			
12.21					23.58	49.75	7.27
12.24		47.6	89	4.24			
12.50					24.44	51.55	7.27
12.53		66.6	77	5.14			
1.09		73.0					

of respirations. It was found, in fact, that *accelerated respirations may occur before any measurable increase in CO_2 tension or hydrogen ion concentration.* From this the conclusion seems justified that the changes in CO_2 tension and pH are not the cause of rapid and shallow breathing which arises after embolism of the pulmonary arterioles and capillaries.

In Experiment 8 the respiratory rate, before starch injection, was 12 to the minute. At this time the pCO_2 was 66.95 mm. and the pH 7.23. After starch injection the rate had risen to 47 but the

pCO$_2$ was 63.42 mm. and the pH 7.25. Similarly with Experiment 14, in the control period the respiratory rate was 23 per minute and the average tidal air 113 cc. Starch injection resulted in a rate of 48 to the minute with a decrease in depth to 89 cc. No significant change, however, had occurred in the CO$_2$ tension or hydrogen ion concentration. The facts of this experiment (No. 14) are supplied in Table VI and in the appended protocol.

Protocol of Experiment 14. (See Table VI.)

Female, mongrel hound. Weight 12.2 kilos.
9.55. 3.36 gm. barbital-sodium, dissolved in 25 cc. distilled water, injected into the left leg vein.
10.05. Dog quiet, snoring. 10.30. Dog quiet, insensitive. Cannulated right jugular vein and right femoral artery.
11.00. Respirations 27. Pulse 205. Rectal temperature 38.4°C. Corneal reflex present.
11.08 Additional 5 cc. 5 per cent solution of barbital-sodium.
11.25. Respiratory mask adjusted to muzzle. 11.30. Breathing 95 per cent O$_2$ from Douglas bag. 11.41. Connected with Tissot spirometer.
11.43. First respiratory period. Expired air collected for 5 minutes. Total volume 12.6 liters. 118 respirations.
11.49. 25 cc. bright red arterial blood drawn from right femoral artery.
11.51. Second respiratory period. Expired air collected for 5 minutes. Total volume 13.9 liters. 116 respirations.
12.00. Injection of 2 per cent starch suspension begun. Respirations continuously counted.
12.01. Respiratory rate 26.
12 03. Total of 50 cc. starch suspension injected.
12.04. Respiratory rate 24.
12.09. Total of 65 cc. starch suspension injected. Respiratory rate 35.
12.14. Third respiratory period. Expired air collected for 5 minutes. Total volume 19.8 liters. 211 respirations.
12.21. 25 cc. bright red arterial blood drawn from right femoral artery.
12.23. Fourth respiratory period. Expired air collected for 5 minutes. Total volume 21.2 liters. 238 respirations.
12.46. Respiratory rate 61 per minute.
12.50. 25 cc. bright red arterial blood drawn from right femoral artery.
12.52. Fifth respiratory period. Expired air collected for 5 minutes. Total volume 25.7 liters. 333 respirations.
1.00. Rectal temperature 38°C.
1.01. Respiratory rate 72.
1.09. Respiratory rate 73.

At this time the experiment was brought to a conclusion and the animal used for another purpose.

SUMMARY AND CONCLUSIONS.

1. The production of multiple emboli of the pulmonary capillaries and arterioles results in rapid and shallow breathing which may be associated with anoxemia, but is not dependent for its occurrence upon anoxemia.

2. Similarly there may occur an increase in the partial pressure of CO_2 in the blood as well as an increase in hydrogen ion concentration.

3. These changes must be regarded as the result of the impaired pulmonary function.

4. They are not, however, the cause of the rapid and shallow respirations, since the abnormal type of breathing may occur without the attendant blood changes.

5. The characteristic type of response to increase in CO_2 tension is an increased rather than a decreased depth of respiration.

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THE EFFECT OF MULTIPLE EMBOLI OF THE CAPILLARIES AND ARTERIOLES OF ONE LUNG.

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INTRODUCTION.

The striking fact (Dunn (1), Binger, Brow and Branch (2)) that obstruction to the pulmonary arterioles and capillaries leads to a marked disturbance of the respiratory mechanism with the development of rapid and shallow respirations, and an increase in the minute volume of pulmonary ventilation, presents a pathological state which we have attempted to analyze further. Interest in this effect lies partly in its clinical application to such conditions as lobar pneumonia in which accelerated respirations are an important physical sign; and partly in the opportunity for investigating the difficult problem of respiratory rhythmicity and some of the factors influencing it. The reasons for thinking that an analogy exists between the rapid breathing due to multiple emboli of the arterioles and capillaries and the rapid breathing occurring in lobar pneumonia are several. In the first place in both conditions rapid breathing may occur independently of anoxemia. In the animal with multiple emboli this fact has been experimentally shown (2). And we have frequently observed patients with pneumonia continuing to breathe at the rate of 40 or more to the minute even after oxygen want has been relieved by oxygen administration. The occasional persistence of rapid respirations after crisis, when pulse and temperature have returned to normal, has suggested that the stimulus for accelerated respirations may be a local one resulting from the pulmonary lesion. A probable occurrence of capillary fibrinous thrombi has been described in pneumonic lungs by Kline and Winternitz (3), and that obstruction to the circulation may

occur in the consolidated lobe is known. This last fact again suggested a possible similarity in causes operating to produce similar effects.

Moreover, it has been found in experimental pneumonia produced in dogs by intratracheal insufflation of cultures of *B. friedländeri* (Porter and Newburgh (4)) that the dyspnea which resulted could be checked by sectioning the vagi or blocking vagal impulses with cocaine. A similar slowing of accelerated respirations has been demonstrated for the tachypnea resulting from multiple experimental emboli. In this condition either vagal section (1) or vagal freezing (2) immediately brings about slow, deep breathing.

Quite aside from its immediate application to disease, a study of changes in respiratory rate has appeared important to us from the point of view of the nervous control of respiration. Of this subject not much is known. We know that the so called respiratory center sends out impulses which vary in strength and frequency, according to certain changes, physical and chemical, occurring either in the blood stream or in the center itself. And we know that the character of breathing, *i.e.* rate and depth, is influenced in some manner by impulses travelling along the vagus nerves (Le Gallois (5), Hering and Breuer (6), Head (7)). That these impulses are of a centripetal nature may be assumed from the studies of Einthoven (8) in which he demonstrated electrical changes in the thoracic end of the cut vagus occurring synchronously with changes in distension of the lungs. Whether there are centrifugal vagal impulses important to the control of respiration is not known. The vagus nerves in the dog, more than in the cat and rabbit, are not pure nerves, but are colonies containing sympathetic and depressor fibers as well. These nerves supply both heart and lungs. Cutting and freezing experiments are therefore necessarily varied in their effects and difficult to interpret.

The perhaps undue emphasis which we have placed on respiratory rate rather than on the minute volume of pulmonary ventilation lies in our interest in the nervous mechanism, where change in rate must represent a reversal in impulse occurring either peripherally in the breathing apparatus or in the respiratory center.

We must again emphasize the great difficulties introduced by the use of anesthetics. One is indeed caught between the horns of a

dilemma. To study the respiration in animals without an anesthetic is often impossible when concerned with changes involving the circulation in the lungs. We have previously (2) alluded to the effects of emotional and other adventitious stimuli which may confuse an experimental procedure. And yet equally confusing may be the sometimes excitatory, sometimes depressing influences of the anesthetic used to obviate the other effects. We have varied our technique, using luminal sodium by stomach tube, or barbital-sodium given intravenously or again ether with sterile technique and survival, in animals previously trained to lie still and breathe quietly. In the interpretation of results we have had constantly to keep in mind the effects of anesthetics.

The characteristic gross and microscopic pathology of the lungs of dogs in which multiple emboli of arterioles and capillaries have been produced by injection of suspensions of starch cells is congestion, edema and atelectasis. This is associated with a diminution in lung volume as determined by a measurement of the functional residual air (2). It seemed not improbable that these changes resulted in a decrease in elasticity of pulmonary tissue producing a shallow tidal air and thus, through the mechanism of the Hering-Breuer reflex, a rapid respiratory rate. Could the phenomenon be reproduced by causing similar lesions in one lung? If so, this would tend to establish more clearly the analogy with rapid and shallow breathing as it occurs in pneumonia. Experiments were planned with an eye to answering this question. It must be stated in advance that because of the technical difficulties involved and the necessary operative trauma the answers were not as unequivocal as was hoped.

EXPERIMENTAL.

Experiment 1. Effect of the Injection of Starch Suspension into One Lung.— A female dog, weighing 7 kilos, was given by slow intravenous injection 2.50 gm. barbital-sodium dissolved in distilled water, or 0.34 gm. of the drug per kilo of body weight. Within ½ hour the dog was quiet and relaxed, and breathing regularly at the rate of 13 respirations per minute. The animal was then intubated and artificial respiration started by the intratracheal insufflation of an interrupted current of air. With the dog lying on its right side the thorax was opened by incising the skin, muscles and pleura in the 4th left intercostal space. The ribs were separated by mechanical retractors, and with very little blunt dis-

section the artery to the left lung was freed. By a method to be described below a suspension of starch grains (sufficient ordinarily to produce rapid and shallow breathing when injected intravenously) was injected directly into the artery supplying the left lung. Thereupon the animal's thorax was closed by approximating the 4th and 5th ribs by three stout ligatures, care being taken to distend the lungs and drive all air out of the pleural space before closing the chest. The muscle and skin layers were next repaired by suturing, the dog turned on its back and allowed to breathe spontaneously after removing the tracheal tube. To avoid the complications of anoxemia a stream of oxygen was blown through a funnel placed over the dog's muzzle. In this animal the preoperative respiratory rate varied from 16 to 20 breaths per minute. The postoperative rate, after the temporary acceleration had subsided, varied from 24 to 21. Following a steady respiratory rate at this level for 25 minutes the dog was killed by the intravenous injection of 20 cc. of a saturated solution of magnesium sulfate, and autopsied. The abdominal cavity and its viscera were normal. On opening the thorax the left lung, containing the starch grains, was mottled and paler than the right, which appeared normal. Frozen sections of the lungs, stained with Lugol's solution, showed the four lobes of the right lung to be free from starch grains, whereas in the small vessels of the left lung, especially in the upper and ventral lobes, they were thickly distributed.

From this experiment the following tentative conclusion can be drawn: Embolism of the capillaries and arterioles of one lung (the left) does not result in rapid and shallow breathing, when the other lung is functioning normally. The phenomenon is therefore not of an irritative nature due, solely, to the presence of the embolic material in one lung.

There is one important criticism to this experiment which obviously makes it inconclusive, namely: Was there actually enough embolic material introduced into the left lung to produce rapid and shallow breathing? It has repeatedly been observed that with intravenous starch suspension injections into the intact, unoperated animal a certain volume of suspension is necessary before accelerated respirations start. This fact was interpreted as indicating that the phenomenon was in some manner related to the degree of obstruction to the pulmonary circulation rather than the result of irritative, local stimuli set up in the lungs.

*Method of Injecting Starch Suspensions Directly into the Artery
Supplying One Lung.*

As this method may prove to be of use to others it seems worth describing in detail.   The method has the advantage of allowing the injection to proceed without clamping the artery or interfering with the circulation in the lung.   The injection apparatus consists of a graduated burette to which about 10 inches of rubber tubing are attached.   The tubing passes through a du Noüy* pump, which operates by rotating a wheel, designed to compress and decompress alternately
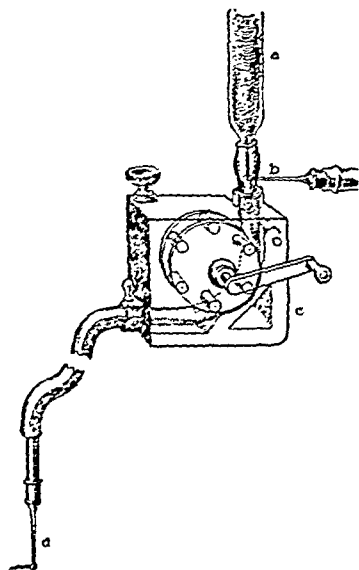
TEXT-FIG. 1.   Apparatus for injecting suspensions into the pulmonary artery or its branches.   *a*, burette; *b*, hollow needle through which a stream of air is blown to keep suspended particles from settling; *c*, du Noüy pump; *d*, bent needle with ball of solder in place.

the rubber tubing, thereby ejecting the fluid which enters the tubing from the burette.   The speed and force of injection can be varied at will by varying the number of rotations of the wheel.

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* This very useful pump, which was devised by Dr. du Noüy, of The Rockefeller Institute, has not previously been described in the literature.   It was manufactured by the Central Scientific Company.   We are indebted to Dr. du Noüy for its use.

The distal end of the rubber tube connects with a No. 21 gauge Luer needle 3 cm. long. 1 cm. from its point this needle has been bent at right angles by heating to a dull red, bending with a pair of nippers and then retempering. A small ball of solder is fused on to the needle at the right angle bend. Before thrusting the needle point through the arterial wall a small piece of muscle is "baited" on to the needle in much the same manner as baiting a fish hook. The ball of solder keeps the fragment of muscle from sliding around the bend and up the shank of the needle. This muscle fragment serves the double function of a flap valve over the puncture wound and of supplying tissue extracts which act as coagulants. With this equipment the arterial wall can be punctured with little hemorrhage and the needle kept in place for 15 to 20 minutes or more, without interfering with the flow of blood through the vessel. On removing the needle, the bleeding which usually occurs at the puncture wound can be controlled by the application of a fragment of muscle and cotton pledgets moistened in warm saline. Text-fig. 1 illustrates the burette, pump and needle used for injecting suspensions into the pulmonary artery or its branches.

Experiment 2.—An important corollary to the foregoing experiment, and one free from the criticism applicable to it, is the following, in which a dog, similarly anesthetized with barbital-sodium, was prepared for the experiment by ligating the left branch of the pulmonary artery. After the thorax had been closed, the animal was allowed to breathe oxygen. The respiratory rate at this time was 10 to the minute. A starch suspension of the same strength as in the previous experiment was then introduced into the circulation through a cannula in the right jugular vein. Since the left branch of the pulmonary artery had been ligated, all the starch entered the vessels of the right lung. The result was rapid and shallow breathing, the rate reaching a level of 58 to 60 per minute, where it remained for 1 hour before the animal was killed. The absence of anoxemia, as a contributing cause to the rapid breathing, was established by analysis of the dog's arterial blood at the close of the experiment, the percentage oxygen saturation being 100.

At this point, the tentative conclusion can be drawn that emboli in the capillaries and arterioles of one lung do not produce rapid and shallow breathing when the circulation through the other lung is normal. Or, in other words, the presence of embolic material, such as starch grains in one lung, does not excite this change in respirations. When, however, the circulation to the other lung is cut off by ligating its artery, emboli of capillaries and arterioles of one lung do produce rapid and shallow breathing in spite of the absence of oxygen want. Under the conditions of the experiment, the "embolized" lung adequately satisfied the animal's oxygen needs, but, even so, respirations assumed a definitely abnormal and accelerated character.

In an effort to surmount our own objections to the first experiment of this pair (Experiment 1), a new approach was devised.

A method was employed by which the left branch of the pulmonary artery could be occluded after the thorax had been closed and while the animal was breathing naturally. The occlusion was accomplished by inflating a pneumatic rubber cuff which had been previously placed about the left branch of the pulmonary artery. The method is given in detail in another paper in this *Journal* (9). Starch suspensions were then injected intravenously so that the emboli lodged in the vessels of the right lung. The left branch of the pulmonary artery was then released. By so doing, blood was allowed to circulate through the left lung after emboli, sufficient to produce rapid and shallow breathing, had lodged in the right lung. Under these conditions, does reestablishing the circulation through the left lung restore the respiratory rate to its original level? It should be stated that in these experiments too, anoxemia was prevented by oxygen inhalation.

The results of this series of experiments were variable. In some, reestablishing the circulation to the left lung (after production of rapid and shallow breathing by intravenous injection of starch granules which had lodged in the capillaries of the right lung) was accompanied by a definite slowing of respiratory rate. In others, the rate remained accelerated. In only one experiment (No. 3), was the respiratory rate restored to its slow, preoperative level.

In this experiment, the dog breathed at the rate of 16 per minute before it was operated upon, and the arterial blood was 97 per cent saturated with oxygen. The thorax was then opened and a pneumatic cuff placed about the left branch of the pulmonary artery. After closing the thorax, and allowing the animal to breathe naturally, the rubber cuff was inflated through a tube which projected through the chest wall. Inflation of the cuff completely shut off the flow of blood to the left lung. This resulted in no increase in respiratory rate. Indeed, at this stage of the experiment, the dog was breathing 13 times per minute. A starch suspension was thereupon injected intravenously, with the result that the respirations became shallow and accelerated to the rate of 61 per minute, despite the fact that the arterial blood was still 95 per cent saturated. The obstruction to the left branch of the pulmonary artery was next released by allowing the cuff to deflate, with the result that within 4 minutes the respiratory rate had dropped to 11 per minute.

This experiment is a clean-cut one and is more susceptible of interpretation than the others of this series. The dog breathed normally with only one lung, the right. When small emboli were introduced into the right lung, the respiratory rate accelerated to nearly 5 times

its previous rate. When, however, circulation was reestablished in the left lung, the respiratory rate returned to normal. Clearly, then, from this experiment too, one could conclude that emboli in the capillaries of one lung alone (the right) do not produce rapid and shallow breathing, unless the other lung is thrown out of the circulation, or, (as is suggested below), otherwise structurally damaged. That this last may account for the variable results referred to above is suggested by the following observations. In several experiments, the left lung, which had been temporarily deprived of its arterial blood, was, at autopsy, found to be bright red in color, instead of the normal salmon-pink. We know that depriving an organ of its blood supply may result in hyperemia from dilated capillaries, when the blood is again admitted (10). It is our opinion, though this fact is not definitely established, that in those animals in which accelerated respirations persisted after reestablishing the circulation to the left lung, some such capillary damage had been produced.

Experimental evidence for this statement is supplied by histological studies of the left lungs of Dogs 3 and 4. In Dog 3 (the experiment cited above in which the rate returned to normal on release of the cuff) the microscopic anatomy of the left lung is essentially normal. In Dog 4, however, in which the left lung, at autopsy, was noted to be bright red and granular in appearance, the respiratory rate remained at 61 per minute even after release of the obstruction to its artery. Here the microscopic picture is definitely abnormal, the thickness and distortion of capillaries being plainly visible. We reproduce photomicrographs of two typical regions of the left lungs of these two dogs which show clearly this difference in them (Figs. 1 and 2).

It should be stated that the variable behavior of different animals could not be correlated with variations in the percentage of oxygen saturation of the arterial blood, nor in the CO_2 tension or pH of the blood (11). Since this is true, we have not considered it worth while to present these data in this publication.

<div align="center">DISCUSSION.</div>

When a suspension of potato starch grains is intravenously injected into a dog, the grains lodge in the finer arterioles and capillaries of the lungs. No change in the dog's respirations occurs until a certain, somewhat variable (in different animals) dose has been given. The

dog then develops rapid and shallow breathing, the respiratory rate often reaching 60 or more per minute. This phenomenon has interested us, partly because of an apparent analogy to the accelerated respirations seen clinically in cases of lobar pneumonia, partly because we believed that an understanding of this condition might throw some light on the problem of the rhythmicity of respiratory movements, and in particular upon the so called Hering-Breuer reflex.

Previous work has not revealed the cause of this abnormal type of breathing. It has not been correlated with (1) changes in arterial or venous blood pressures, (2) changes in percentage saturation of the arterial blood with oxygen, (3) changes in carbon dioxide tension or pH of the blood. It was found, however, to be associated with a reduction in lung volume, as expressed by measurement of the so called functional residual air, or that volume of air remaining in the lungs at the end of a quiet, normal expiration. And it was found that the characteristic pathological picture associated with this abnormal type of breathing was congestion, atelectasis and edema of the lungs.

The present study was undertaken to discover whether similar emboli introduced into one lung would result in a similar change in breathing. To accomplish this, that is, introduction of emboli into one lung, we had to resort, not only to the use of anesthetics, but to difficult and drastic operative procedures. Such procedures, of course, have drawbacks, and we have previously dwelt on the care necessary in interpreting results in the face of them.

Experimental evidence here presented appears to us to justify the conclusion that emboli lodged in the arterioles and capillaries of one lung produce an accelerated type of breathing only when the other lung has been excluded from the circulation by ligating or clamping its artery, or when the capillaries of the other lung have been abnormally distended by depriving them of blood for a period and then again admitting the blood.

The phenomenon would, therefore, appear to be in some manner related to the condition of diminution of the pulmonary vascular bed, to congestion in the lung or to resistance to the flow of blood through the lungs.

SUMMARY AND CONCLUSIONS.

1. Injection of a suspension of potato starch cells into the left branch of the pulmonary artery, in quantity sufficient ordinarily to give rise to markedly accelerated respirations, resulted in no change in respiratory rate.

2. A method for injecting substances into the pulmonary artery or its branches without interfering with the blood flow to the lungs has been described.

3. Injection of similar material into one lung when the other is excluded from the circulation either by ligation or by temporary clamping does give rise to rapid and shallow breathing (from a rate of 10 to 15 per minute to one of 60 or over) identical in character to that brought about by introducing emboli into both lungs.

4. A method for clamping and releasing the pulmonary artery or its branches in a dog breathing normally with closed thorax has been devised. This is described in detail in another paper.

5. After rapid breathing has been initiated by the effect of emboli lodged in the arterioles and capillaries of the right lung, reestablishing the circulation in the other lung by releasing the clamp on its artery may or may not restore the respiratory rate to its original, normal level.

6. This discrepancy in results has not been correlated with any difference in oxygen saturation of the arterial blood, or in carbon dioxide tension or pH of its plasma.

7. It is, however, believed to be related to the gross and micro-scopic anatomy of the lung of which the artery has been temporarily clamped. Photomicrographs are published, showing in one dog (No. 3), in which the respiratory rate returned to normal, a normal histological picture of the left lung, and in another dog (No. 4), in which the rate remained rapid after release of the clamp, a picture characterized by congestion and dilatation of arterioles and capillaries.

8. The fact that accelerated respirations result from emboli in the pulmonary capillaries and arterioles only after a certain quantity of material has been introduced, and the fact that emboli in one lung do not occasion accelerated respirations unless the circulation through the other lung is occluded or abnormal, leads us to the conclusion that

the phenomenon is not an irritative stimulus due to foreign bodies, but is in some manner related to (*a*) diminution of the pulmonary vascular bed, (*b*) resistance to the blood flow through the lungs or (*c*) congestion or dilatation of the arterioles and capillaries of the lungs.

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EXPLANATION OF PLATE 23.

FIGS. 1 and 2. Photomicrographs of sections of lungs from Experiments 3 and 4. × 130.

FIG. 1. Experiment 3. Section from left upper lobe. The histological picture is essentially normal.

FIG. 2. Experiment 4. Section from left upper lobe. The picture shows marked engorgement and tortuosity of alveolar capillaries.

Both preparations were made with similar technique, the dogs being killed by the intravenous injection of a saturated solution of magnesium sulfate. The lungs were fixed by immersion in Helly's fluid, after they had been distended *in situ* by the intratracheal injection of about 1 liter of Helly's fluid.
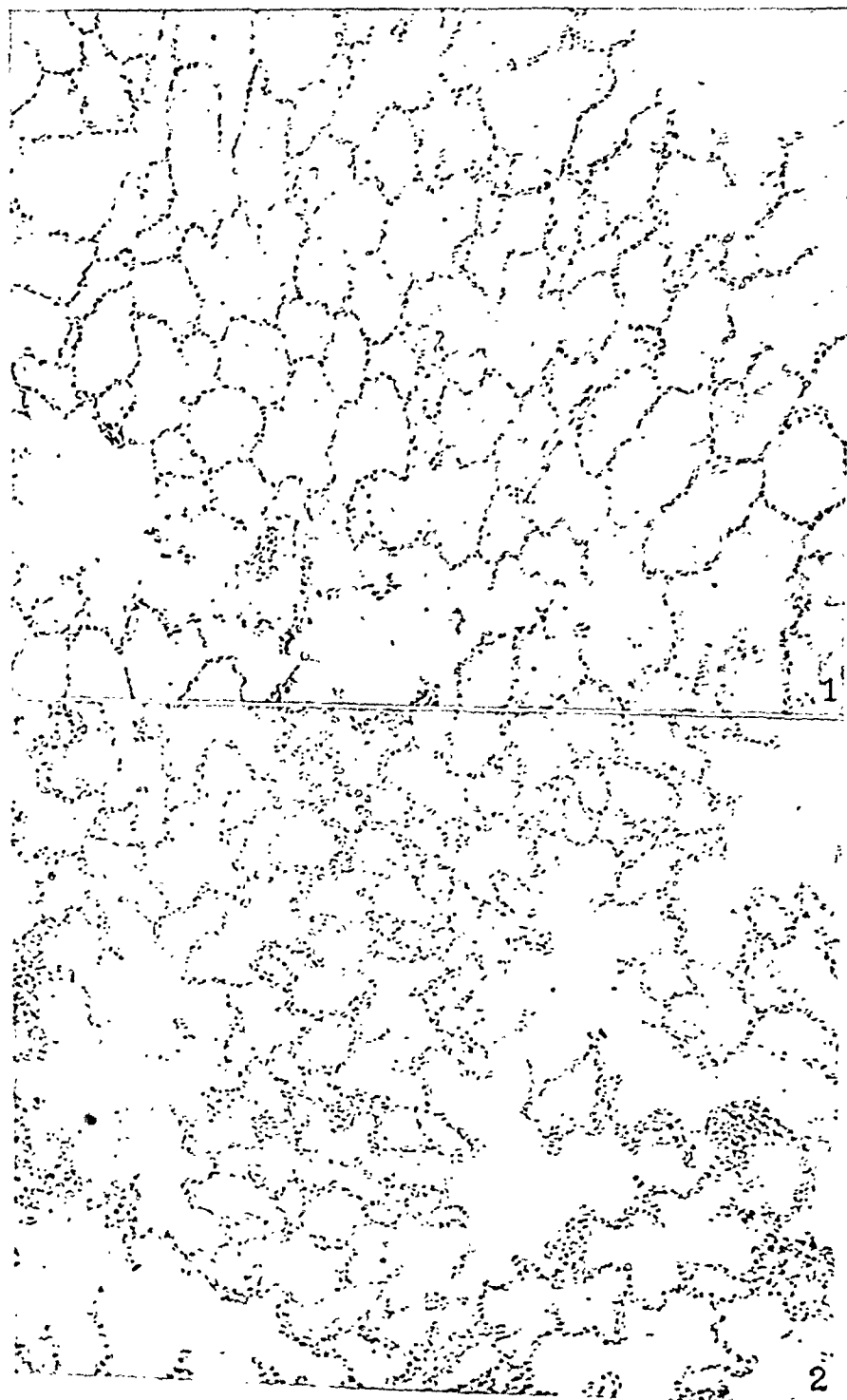
OBSERVATIONS ON RESISTANCE TO THE FLOW OF BLOOD TO AND FROM THE LUNGS.

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PLATES 24 AND 25.

INTRODUCTION.

Previous studies (1–4) have led us to the view that the cause of rapid and shallow breathing which results from multiple emboli in the arterioles and capillaries of the lungs must be sought for in the secondary vascular changes following embolism. Neither the presence of the embolic material *per se* nor the chemical changes found in the blood can be regarded as the direct cause of the modified type of breathing. The phenomenon appears to be related in some manner to the diminution of the pulmonary vascular bed, to the resistance to the flow of blood through the lungs or to the state of congestion and edema of the lungs. It has already been shown (2) that obstruction to the larger branches of the pulmonary artery by the intravenous injection of seeds of various sizes results in a condition quite different from that due to capillary obstruction. The functional abnormalities in breathing thus produced were found to result wholly from the condition of oxygen want. That blocking the capillary bed should have a different effect from blocking some of the larger branches of the pulmonary artery is not hard to understand. The first might be described as an "effective blockade" in which the blood passing through the lesser circulation must necessarily meet resistance. In blocking some of the larger branches, however, the blood has an alternative route to follow, and such resistance may not be encountered.

The probability that resistance to flow of blood through the lungs would result in heightened pressure in the pulmonary artery and perhaps the right heart was borne out by the work of Haggart and Walker

(5) and of Wiggers (6). It occurred to us that such pressure changes might result in stretching of the walls of the pulmonary artery or of the right ventricle and that this might induce afferent stimuli which could occasion reflex changes in respirations. No experimental evidence for this assumption, however, can be adduced from this work, and the results here published, though perhaps negative from the point of view of explaining the origin of rapid and shallow breathing, are of themselves of interest, we believe, and have helped us with an understanding of the problem.

<div align="center">EXPERIMENTAL.</div>

Dilatation of the Pulmonary Artery and Right Ventricle Following Obstruction to the Arterioles and Capillaries of the Lungs.

Without indulging in the artificial conditions necessarily prevailing in cardiac oncometry an effort was made to determine what changes occurred in the state of dilatation of the pulmonary artery and right ventricle after obstructing the flow of blood through the lungs by the intravenous injection of a suspension of potato starch grains. This was done by careful x-ray photographs made of the heart during injection of starch suspensions. A comparison of the photographs made under uniform conditions before and after the onset of rapid and shallow breathing shows a marked area of bulging at the base of the heart on the left side and a flaring out of the shadow cast by the right side of the heart. This was clearly shown in two experiments (Nos. 1 and 2). The x-ray photographs in Experiment 1 taken before and after the onset of rapid breathing following starch injection are reproduced in Fig. 1. The bulge at the left side of the base is plainly to be seen, as well as the flare in the shadow cast by the right side of the heart. At the time of the first picture the dog was breathing at the rate of 16 per minute; at the time of the second the respiratory rate was 64. The area of the cardiac shadow, measured by a planimeter, averaged 43.4 sq. cm. before the injection of starch as compared with 48.4 sq. cm. after. And the maximum transverse diameter of the cardiac shadow before embolism was 6 cm. as compared with 6.7 cm. after.

Like results were obtained in Experiment 2, in which x-ray photographs showed similar bulging at the left base of the heart and flaring

of the right border. By taking a series of x-ray plates during the experiment it was shown that these changes occurred synchronously with the acceleration of respirations. These facts are made plain by the two curves shown in Text-fig. 1 in which the lower represents
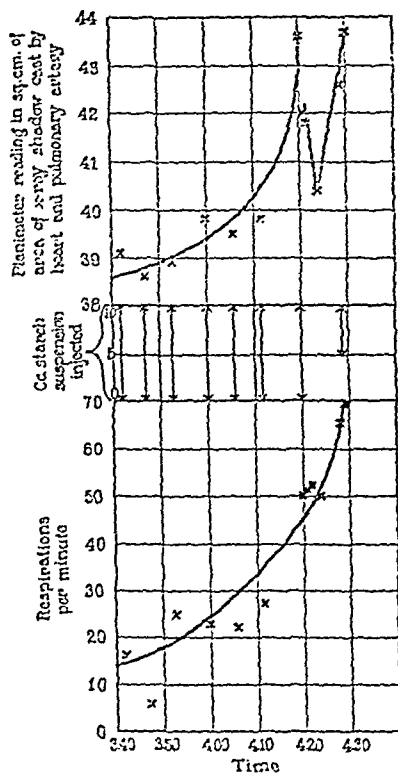
TEXT-FIG. 1. Lower curve: Ordinates, respirations per minute; abscissæ, time. Upper curve: Ordinates, area in sq. cm. as measured by a planimeter of x-ray shadow cast by the heart and pulmonary artery; abscissæ as in lower curve. The length of each arrow represents the volume of starch suspension injected at the time specified in the abscissæ.

respiratory rate per minute at the times specified in the abscissæ, and the upper, the area of the cardiac shadow in sq. cm. as measured by a planimeter. The arrows represent the times at which suspensions of starch were intravenously injected. The two curves will be

seen to vary synchronously and in the same direction. The break at the end of the upper curve may be regarded as a temporary recovery of muscular tone overcome by the injection of an additonal 5 cc. of starch suspension.

The planimeter measurements recorded here and in Experiment 1 were made by tracing the whole dense shadow cast by the heart, including the bulge at the left base which was suspected of being caused by the dilated pulmonary artery. By tracing the ventricular shadow alone from arbitrary points after the method of Levy (7) and Stewart (8), it was found that the increase in area was far less marked than it was when the bulge was included in the tracing. Direct inspection of the photographs shows the heart after embolism to be wider and shorter than before.

By carefully splitting the sternum without loss of blood in a dog[*] breathing at the rate of 56 per minute following starch embolism (Experiment 3) and maintaining the same respiratory rate by interrupted, intratracheal insufflation, after the chest had been opened, it was shown beyond doubt that the shadow of the bulge at the left base of the heart previously alluded to was cast by a greatly dilated pulmonary artery. This structure stood out a tense, bulging, sausage-like mass, three or four times its normal size.

The experimental facts thus far presented, we believe, prove that *obstruction to the capillaries and arterioles of the lungs results in an increase in the area of the x-ray shadow cast by the heart, and that this increase is the result partly of a widening of the transverse diameter of the ventricles, but chiefly of a marked bulging of the pulmonary artery. The changes in the heart and pulmonary artery were shown to occur synchronously with the acceleration of respiratory rate.*

The hypothesis on which we proceeded, and which was found to be faulty, was that the dilated pulmonary artery sent out impulses which reflexly accelerated respirations. Though the results of these experiments are in a sense negative, by eliminating false causes, they largely clarify our problem.

Experiments were devised with an eye to obstructing the blood flow to the lungs proximal to the pulmonary parenchyma itself so that

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[*] The animal was anesthetized with barbital-sodium.

any changes in breathing could be attributed wholly to the cardio-vascular effect, unclouded by pathological changes in the lungs. Two methods were used to accomplish this end: (1) the exposed heart method of Drinker; (2) a new method which will be described in detail below.

Experiments with the Drinker Exposed Heart Preparation.

By the technique devised by Drinker (9) the heart of a cat was exposed through a window cut into the sternum and the thorax closed by stitching the split pericardium to the edges of the window. The heart thus everted and supported on its pericardial sling, could be observed and its great vessels manipulated at will while the animal breathed without the aid of artificial respiration. To obstruct the flow of blood through the pulmonary artery, or one of its branches, a clamp and ligature were placed about it, after the method described by Haggart and Walker (5), in such a manner that progressive tighten-ing of the ligature resulted in progressive diminution of the calibre of the vessel until it was completely occluded.

This experiment was performed on two cats anesthetized with barbital-sodium. In one of them (No. 4) the clamp was placed on the left branch of the pulmonary artery, in the other (No. 5) on the main trunk of the artery. Since the results of these two experiments are in complete agreement with those of Haggart and Walker (5) they will not be given in detail. In Experiment 4, in which the left branch of the pulmonary artery was suddenly occluded, no change occurred in the animal's respirations. In Experiment 5, in which the main trunk of the pulmonary artery was gradually compressed, no change in respiratory rate or depth occurred until a point was reached when respirations ceased entirely.

Because all of our previous studies on the effect of pulmonary embolism on breathing had been made on dogs, it was determined to repeat the last two experiments on dogs instead of cats. This, how-ever, brought us face to face with a real difficulty. The Drinker ex-posed heart preparation, though admirable in the cat, cannot be used satisfactorily in dogs because of the depth of the dog's thoracic cavity, and the difficulty to effect a tight closure between the pericardial edges and the chest wall. Moreover, it seemed desirable to devise a

method in which the thoracic cavity was not restricted in size and one which could be used, if need be, in experiments made without anesthesia, on animals previously prepared by sterile, survival operations.

The method finally achieved was the following: A thin walled, flat rubber bag measuring 1.8 × 5.5 cm. communicating with a 30 cm. length of rather heavy walled rubber tubing (3 mm. in cross-section with a 2 mm. bore) was passed under the previously exposed and freed pulmonary artery.* By surrounding this rubber bag or cuff with a stocking of closely woven silk, to the four corners of which ligatures were attached, the rubber bag could be fastened in position about the vessel. The silk covering prevented overdistension of the bag on inflating it, and assured a uniform compression on the vessel, similar to that produced by the ordinary blood pressure cuff. Inflation of the bag was made through the patent end of the rubber tubing. The pressure in the cuff could be accurately controlled by the use of a mercury manometer. Fig. 2 illustrates the bag in place before the ligatures were fastened. When this is done, the bag forms a closely fitting cuff surrounding the vessel wall. Inflation immediately compresses the vessel. It was found that a pressure of 150 mm. Hg was more than sufficient to obliterate the lumen of the artery. As has been said, the method could be used in survival experiments in which the pulmonary artery or one of its branches could be occluded without the use of anesthetics, and after the effect of operative trauma had subsided. Fig. 3 is a photograph of such a survival experiment (No. 6) to be referred to below. The rubber tube, projecting through the chest wall, through which the bag is inflated, is shown in the picture.

Effect on Respiration of Compression of the Left Branch of the Pulmonary Artery in the Anesthetized Dog.—Dogs anesthetized by the intravenous injection of barbital-sodium were operated on, with the aid of artificial respiration, an incision into the pleural cavity being made through the 4th left interspace. Spreading the 4th and 5th ribs gave free access to the left branch of the pulmonary artery after the upper and middle lobes of the left lung had been carefully retracted downward and packed out of the field. The left branch of the pulmonary artery was surrounded by the pneumatic cuff described above and the chest wall closed by approximating the spread ribs with three stout ligatures after proper distention of the lungs. In closing the thorax, the free end of the rubber tube which communicates with the cuff was permitted to project through the chest wall.

* When one of the two main branches of the pulmonary artery was compressed. a somewhat smaller bag was used, *viz.*, 0.8 × 4 2 cm.

The effect on breathing of compressing the left branch of the pulmonary artery was noted in a series of experiments. The results obtained are similar to the cat experiment (No. 4) cited above. Blocking the circulation to the left lung produces essentially no immediate (2 minutes to 2 hours) effect on respiratory rate. Five animals showed a slight drop in rate, -1, -3, -3, -1 and -2, respectively. One animal showed no change. Eight animals showed a slight increase in rate, $+2$, $+6$, $+1$, $+5$, $+1$, $+3$ and $+1$. Whereas the average postoperative control rate was 22.5 per minute, the average rate after compression of the pulmonary artery was 24.2   The periods of compression, during which the respiratory rates were observed, varied from 2 minutes to 2 hours. Anoxemia was prevented from arising in these experiments by allowing the animals to inhale oxygen.

From these observations it can be definitely concluded that *restriction of the pulmonary vascular bed by nearly half does not result in rapid and shallow breathing.*

In two of this series of experiments (Nos. 7 and 8), the carbon dioxide tension and hydrogen ion concentration of the serum were studied before and after excluding the left lung from the circulation.

In Experiment 7 there was an increase of nearly 10 mm. Hg in $p\text{CO}_2$ with a corresponding drop in pH from 7.34 to 7.27. In Experiment 8, however, the $p\text{CO}_2$ fell from 54.05 mm. Hg, before clamping the left branch of the pulmonary artery, to 50.35 mm. Hg after, and the pH rose from 7.29 to 7.32. Tables I and II give the analytical data of these two experiments.

No constant effect, therefore, on the CO_2 tension or pH of the serum is to be anticipated from suddenly shutting off the circulation to the left lung.

Effect on Respiration of Gradual Compression of the Main Trunk of the Pulmonary Artery in the Surviving Unanesthetized Dog. Protocol of Dog 6.—(See Fig. 3.) February 3, 1926, a white and black, male setter, weighing 14.5 kilos, was given ether by cone after a preliminary injection of morphine. When the dog was fully relaxed a rubber tube was inserted into the trachea. Artificial respiration and anesthesia were maintained by forcing an air-ether mixture intermittently into the lungs at a pressure of 20 mm. Hg. The dog was placed on its right side, with fore legs extended. Thorax was shaved and skin scrubbed with soap, water and alcohol. The chest was opened by resecting the 5th rib from its costochondral junction laterally for a distance of about 3 inches. By a self-

retaining mechanical retractor the edges of the wound were held apart and the left lung was retracted from the field by a cotton pad moistened with warm, physiological saline solution. A vertical incision was then made in the anterior surface of the pericardium directly over the pulmonary artery, from the point at which

TABLE I.

Experiment 7.

Time	Procedure	Respira-tions	[CO₂]	pCO₂	pH
		per min.	*mM*	*mm.*	
1.41	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	20	27.56	50.40	7.34
1.46	Cuff inflated to 150 mm. Hg				
2.10		26			
2.40		24			
3.25		26	28.45	59.60	7.27

TABLE II.

Experiment 8.

Time	Procedure	Respira-tions	[CO₂]	pCO₂	pH	O₂ capacity	O₂ content	O₂ satura-tion
		per min.	*mM*	*mm.*		*mM*	*mM*	*per cent*
2 14	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	24	26.76	54.05	7.29	8.97	8.88	99
2.17	Cuff on left branch of pulmonary artery inflated to 150 mm. Hg							
2.52		25	26.71	50 35	7.32	9.02	9.10	101
2.52	Cuff deflated							
3.24		25	26.63	49.35	7.33	9.17	9.06	98.8

the pericardium is reflected from the great vessels downward for a distance of 2 inches.

The edges of the pericardium were retracted by temporary ligatures. The pulmonary artery was freed from the aorta by blunt dissection and the cuff tied snugly in place. After approximating the edges of the pericardium, the wound

was closed in layers, the tube connecting with the cuff being led through the incision as far as the platysma muscle, then beneath this muscle, to make its exit through the skin at a point on the dorsolateral aspect of the chest, 3 inches distant from the wound. This was an added precaution taken against the possibility of infection working its way in from the outside. Silk ligatures and sutures were used throughout.

The dog made a splendid recovery and on February 9, 1926, appeared well and acted normally. The only sign of infection was a slight, odorless discharge, which could be expressed about the tube at the point of its exit in the skin. Weight on this date, 13 kilos. The dog had shown a very decided disinclination toward food, but there had been some improvement in this respect over the immediate postoperative period.

February 9, 1926. On four separate occasions the tension in the cuff was gradually raised to the point of producing respiratory failure, without any increase in respiratory rate. Each break was characterized by identically the same symptoms. The dog would suddenly become restless, lift up its head and then begin to whine. This was immediately followed by muscle rigidity, and the respirations, which had become irregular, abruptly ceased. Immediate deflation of the cuff restored the animal to its normal state within a few seconds. The results obtained during the first and second compressions on this date are shown in Tables III and IV.

Similar results were obtained in another dog (No. 10) of this series. In this experiment it was observed at operation that inflation of the rubber cuff resulted in a marked dilatation of that portion of the pulmonary artery proximal to the cuff.

The conclusion can now be definitely drawn that *resistance to the flow of blood to the lungs obtained by sudden or gradual compression of the pulmonary artery, in both the cat and the dog, though it results in dilatation of the pulmonary artery and of the right chambers of the heart, does not give rise to rapid and shallow breathing as do multiple emboli of the pulmonary capillaries and arterioles, but produces practically no change in respirations until syncope and respiratory failure suddenly occur.* From this it is likewise concluded that the markedly accelerated and shallow respirations following embolism of the pulmonary capillaries and arterioles are not the result of a reflex stimulus arising in the dilated heart or pulmonary artery.

Effect of Gradual Compression of the Pulmonary Veins on the Heart and Respirations.—It remained to discover what effect impeding the return of blood from the lungs to the heart would have on the respira-

TABLE III.

Dog 6.

*February 9, 1926.   Sudden Compression of Pulmonary Artery in an
Unanesthetized Dog.*

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
a m.	*mm. Hg*	*per min.*	*per min.*	
11.30	0	19	135	Dog quiet
11.35	0	19	168	
11.36	0	16	124	
11.39	0	18		
11.40	100±	0		Cuff abruptly inflated. Respirations ceased. Cuff released
11.41	0	20		
11.43	0	20	124	

TABLE IV.

Dog 6.

*February 9, 1926.   Gradual Compression of Pulmonary Artery in an
Unanesthetized Dog.*

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
p m.	*mm. Hg*	*per min.*	*per min.*	
3.22	0	20		Dog quiet
3 23	0	22		
3.24	0			Inflation begun
3.25	40	24		
3.30	36	20	152	
3.32	50	21	147	
3.35	47	24		
3.38	62	24		
3.43	74	23		
3.45		24	145	
3.49	85	24	176	
3.52	106	26–0		Respirations ceased
3.58	0	20		

tory rhythm.   Multiple embolism of the pulmonary capillaries and
arterioles, we know, results in a condition of congestion and edema of

the lungs. That this change in structure might be responsible for the altered function was long suspected. Drinker, Peabody and Blumgart (10) showed that compression of the pulmonary veins in the cat resulted in a diminution in lung volume as measured by the volume of air which could enter the lungs under a uniform pressure. This diminution was attributed to the state of engorgement induced in the pulmonary capillaries. When the resistance to the outflow of blood was sufficiently severe and prolonged a condition of pulmonary edema arose. Their work included no functional studies on respiration, as
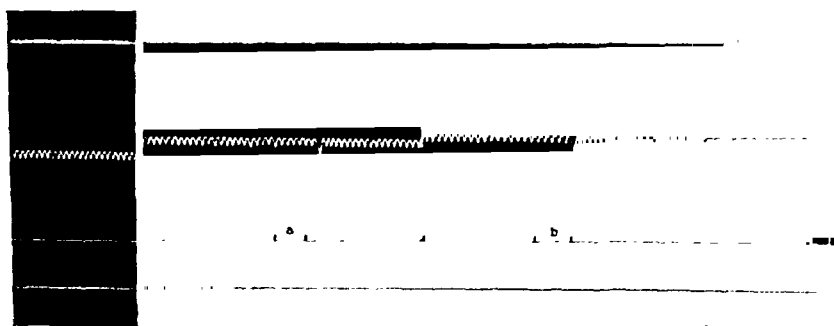
Text-Fig. 2. Graphic spirometer tracing made by cat in Experiment 11. The first portion of tracing was taken during the control period. The second portion of the tracing was taken toward the latter part of the experiment in which the pulmonary veins were being progressively compressed by a ligature. At a and b the ligature was still further tightened. The intervening respiratory rates may be seen in Text-fig. 3.

the animals (cats) were curarized and, therefore, not breathing spontaneously.

We again availed ourselves of the Drinker exposed heart preparation, and in a series of experiments on cats, we gradually compressed the pulmonary veins and observed the effect on the heart and on the respirations. To our surprise exactly the same "all or none" relationship obtained as in compression of the pulmonary artery. Gradually increased pressure exerted by a clamp and ligature on the pulmonary veins leads, after a certain point, to acute dilatation of the heart, followed by cessation of respiration. In no experiment, even when pressure was exerted for many minutes at the point just before respira-

tory failure occurs, did respirations become rapid and shallow. In Text-fig. 2 a graphic tracing is shown of the respirations and heart beat during gradual compression of the pulmonary veins in a cat. The first part of the record is the control period with the ligature in place but slack. It corresponds to the first bracketed zone in Text-fig. 3. The second portion of the record represents heart rate and respirations toward the end of compression corresponding in time to the second bracketed zone in Text-fig. 3. The intervening portion of the graphic spirometer tracing is omitted, but the rate may be seen in Text-fig. 3. During this whole second period the heart was blue and markedly dilated. Respirations will be seen to continue at about uniform rate
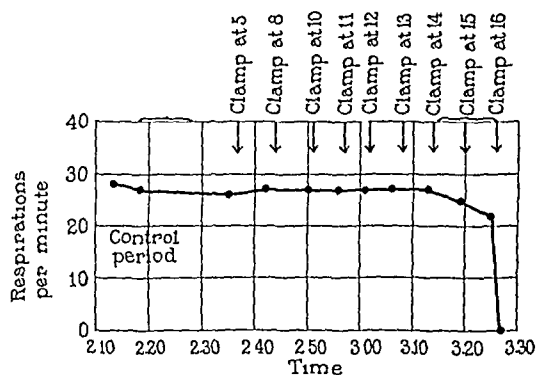
TEXT-FIG. 3 The respiratory rate of the cat in Experiment 11. The bracketed areas in the curve correspond in time to the two sections of the graphic tracing shown here (Text-fig. 2).

and depth until they more or less suddenly cease. This observation was repeated in two other cats in which it was shown that release of the ligature was promptly followed by return of the heart to its normal appearance and the respirations to the control rate and depth. *In no experiments did rapid and shallow breathing develop as the result of gradual compression of the pulmonary veins or, in other words, of increased resistance to the flow of blood from the lungs.* It was, unfortunately, impossible to compress the pulmonary veins in the dog because, as has been said, this animal is anatomically unsuited to the Drinker exposed heart technique, nor is the pneumatic cuff method applicable, so obscurely and closely packed are the veins as they enter the posterior auricular wall.

DISCUSSION.

Since this paper constitutes the last of this series of experimental studies on respiration it may be well, even at the cost of redundancy, to consider again our findings, and what lessons are to be gleaned from them. The work was originally undertaken with a hope of understanding more clearly the mechanism and significance of that type of breathing frequently encountered in patients suffering from lobar pneumonia,—breathing characterized by being both rapid and shallow. Such disordered respiration is known to be accompanied often by oxygen want, and the point has been raised that it may indeed be responsible for oxygen want which, in turn, through its influence on the respiratory center, may perpetuate the disordered type of breathing and lead eventually to the exhaustion and ultimate collapse of the respiratory organ system.

Our work began some years ago with a quantitative study of the degree of pulmonary involvement which occurred in lobar pneumonia (11). By measuring the so called "functional residual air," or that volume of air enclosed in the lungs at the end of a normal, quiet expiration, we arrived at a more or less quantitative estimation of the degree of pulmonary involvement. It was found in general that this function varied with the clinical course of the disease, that after crisis the volume of the functional residual air rapidly increased, and that it remained either stationary or decreased as long as the active infection persisted. No unequivocal correlation, however, could be arrived at between lung volume, so measured, and the type of breathing. Occasionally rapid and shallow respirations were seen to persist for a considerable length of time after the disease process had become arrested. Nor did the state of anoxemia seem to be responsible for the accelerated respirations. In most patients in whom oxygen inhalation relieves the existing state of anoxemia, as determined by oxygen analysis of the arterial blood, rapid respirations still persist.

There seemed to be a hitherto unconsidered element responsible for the disordered breathing and for various reasons this was assumed to be one of a nervous reflex nature. The work of Porter and Newburgh (12) showed that the dyspnea associated with experimental lobar pneumonia in dogs could be checked by sectioning the vagus

nerves or blocking them with cocaine. Dunn (13) discovered the interesting fact that multiple embolism of the pulmonary arterioles and capillaries in goats resulted in very rapid and shallow breathing which could at once be stopped by double vagotomy. Our studies began where Dunn's left off. We found that this phenomenon was inherently related to obstruction of the capillaries and arterioles. When the larger branches of the pulmonary artery were obstructed a similar disorder of breathing resulted, but this was found to be wholly the result of oxygen want and could accordingly be arrested or prevented by oxygen inhalation. Anoxemia of this origin was demonstrated to be due to a relative change in rate of blood flow through the pulmonary vessels. Obstruction of the finer vessels, though often associated with anoxemia, was not in this respect the responsible factor for the disordered breathing, since rapid and shallow breathing persisted even after relief of oxygen want.

What then was the cause of rapid and shallow breathing due to capillary embolism? To this problem we addressed ourselves and it was found far more difficult in its solution than had been anticipated. Indeed a proper understanding of it has depended upon the exhibition of a large amount of so called negative evidence.

We know that capillary obstruction is associated with a diminution in the volume of the "functional residual air" which is probably dependent upon the secondary state of edema, atelectasis and congestion which arises. It seemed reasonable to believe that this pathological condition of the lung, which could give rise to oxygen want, might likewise be responsible for a retention of carbon dioxide, or an increase in the pressure of CO_2 and the concentration of hydrogen ions in the blood. These changes were, in fact, found but that they are not the cause of the disordered breathing is strongly indicated by the fact that rapid and shallow breathing may occur after embolism without any increase in pCO_2 or fall in pH. We know, too, that the actual presence of the embolic material is not responsible for the accelerated breathing in the sense of its being a local irritant, though the inhibitory effect of vagotomy or vagal freezing first made us suspect this to be true. The fact that starch emboli do not produce rapid and shallow breathing solely by acting as local irritants is shown by these two considerations: (1) a considerable amount of embolic mate-

rial may be present without any influence upon the normal respiratory rhythm; (2) emboli may be present in the capillaries and arterioles of one lung sufficient ordinarily to produce rapid breathing without however, any alteration in normal breathing unless the circulation to the other lung is cut off or has been disturbed by a previous period of obstruction. These last facts suggested that the phenomenon was in some manner related to (1) a restriction of the pulmonary vascular bed, (2) resistance to the flow of blood through the lungs or (3) the state of congestion and edema of the lungs. The first two of these possibilities might easily be related to an extra burden on the heart, resulting in an increased pressure in the pulmonary artery and right side of the heart with a subsequent dilatation of these tissues. The demonstra-stration by Levy (7) of an increased heart size in pneumonia was thought perhaps to be a germane phenomenon. It has been shown in this paper that dilatation of the pulmonary artery and right heart does in fact occur when the arterioles and capillaries of the lungs are obstructed. And it was tentatively hypothecated that the dilatation of the pulmonary artery and right heart resulting from obstruction to the finer vessels of the lungs occasioned impulses which reflexly accelerated respirations. Evidence has been brought forth in this paper to show that this hypothesis is erroneous and that reduction of the pulmonary vascular bed, at least by half, produces no change in breathing. Moreover, both resistance to the flow of blood to and from the lungs though followed, when of sufficient grade, by dilatation of the pulmonary artery and right heart, does not give rise to rapid and shallow breathing. The result of such resistance is an "all or none" phenomenon in which respirations continue at their normal rate and depth until they more or less suddenly cease completely.

We are left then to a consideration of the third possibility enumerated above, namely, that rapid and shallow breathing of the kind here described is the result of the particular lesion produced in the lungs, namely, congestion and edema.

How this acts to produce accelerated breathing still remains more or less a mystery: whether directly through irritation of vagal nerve ending; whether as the result of marked encroachment on lung volume; or whether through changes in elasticity of the lung, which to be sure we have not been able to demonstrate in the embolized lung at autopsy.

The first of these three possibilities seems to us at present to be the most likely. It is tempting to theorize but perhaps unwise to befog an already complicated subject by unproven hypotheses. Certainly congestion of the lungs, reduction in lung volume and impaired elasticity of the pulmonary parenchyma are all three intimately associated phenomena, and are present in many of the clinical states in which disordered breathing is a prominent symptom.

SUMMARY AND CONCLUSIONS.

1. Embolism of pulmonary arterioles and capillaries produced by the intravenous injection of starch grains results in a dilatation of the pulmonary artery and the right chambers of the heart. This has been demonstrated both by x-ray studies and direct inspection.

2. The dilatation of the pulmonary artery and heart occurs synchronously with the acceleration of respirations.

3. Dilatation of these structures produced by other means, such as obstruction to the flow of blood to and from the lungs, by gradually clamping either the pulmonary artery (cat and dog) or pulmonary veins (cat) does not, however, give rise to rapid and shallow breathing.

4. The effect of these maneuvers on respiration does not become apparent until respirations suddenly cease.

5. Neither does sudden restriction of the pulmonary vascular bed by clamping the left branch of the pulmonary artery give rise to rapid and shallow breathing, though this procedure may cause an increase in CO_2 tension and in hydrogen ion concentration of the blood.

6. Since rapid and shallow breathing is *not* the result of (1) anoxemia, (2) increased pCO_2 and hydrogen ion concentration of the serum, (3) restriction of pulmonary vascular bed by nearly half, (4) increase in resistance to the flow of blood to and from the lungs, (5) the presence of starch grains in the lungs acting as a local irritant, it must be the result of the secondary pathological changes which occur in the pulmonary parenchyma following embolism.

7. The nature of these changes, congestion and edema, has been discussed elsewhere. Whether they operate directly on nerve endings or through their influence on lung volume and tissue elasticity is not certain.

8. Various important clinical analogies have been emphasized.

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EXPLANATION OF PLATES.

PLATE 24.

FIG. 1, *a* and *b*.   X-ray photograph of dog's heart taken in Experiment 1.   Tube distance 2 meters.   In *a* the dog was breathing 16 times to the minute, the area of the shadow as measured by a planimeter being 43.4 sq. cm.   In *b* after a starch suspension had been injected intravenously, respirations were 64 to the minute, and the area of the shadow is 48.4 sq. cm.

PLATE 25.

FIG. 2. Operative field with pulmonary artery exposed and cuff in place ready for ligation.

FIG. 3. Dog 6, 8 days after operation, showing tube, which communicates with cuff surrounding the pulmonary artery, projecting through the chest wall.
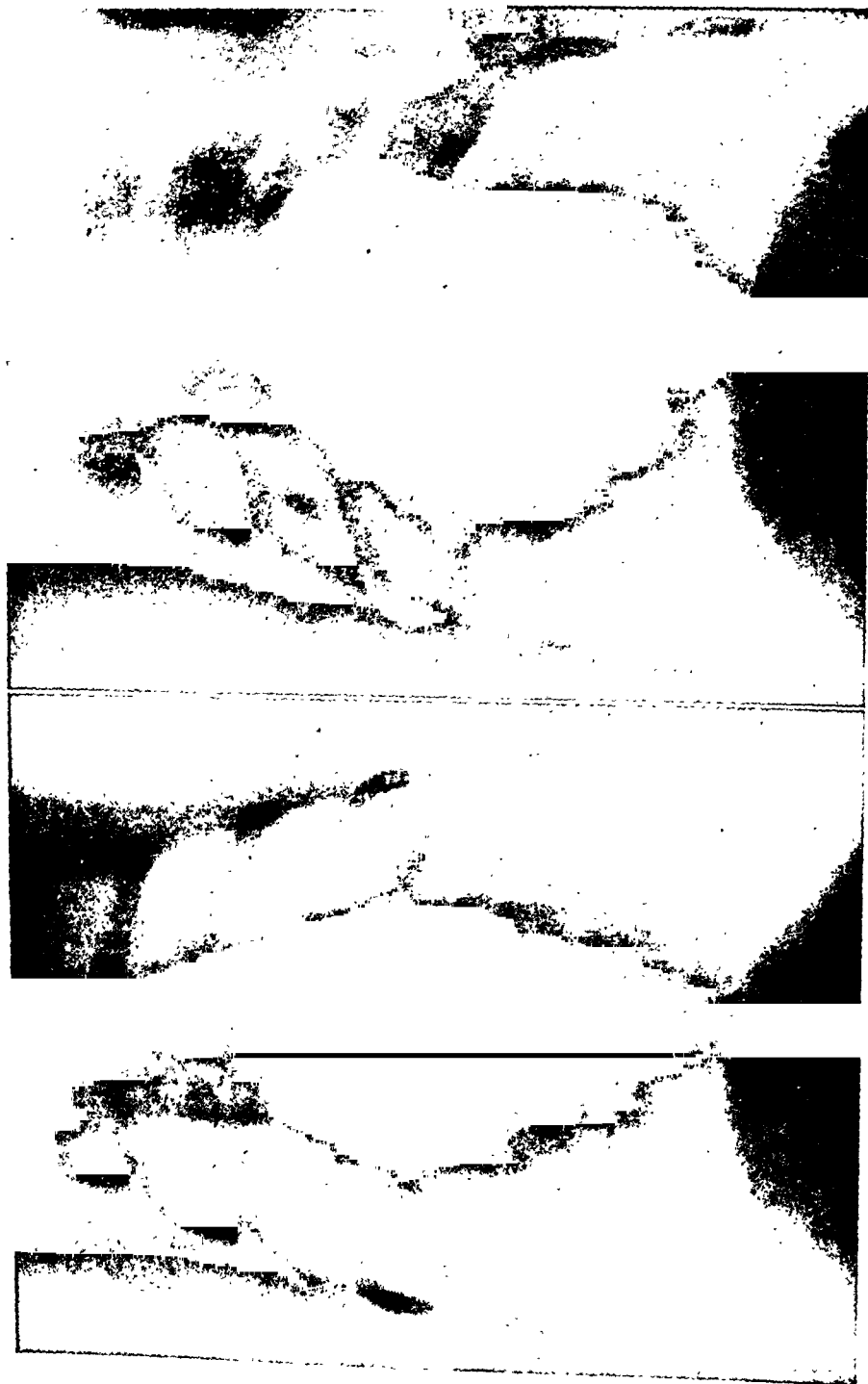
FIG. 1, b.

FIG. 1, a.

(Moore and Binger: Blood flow to and from lungs.)
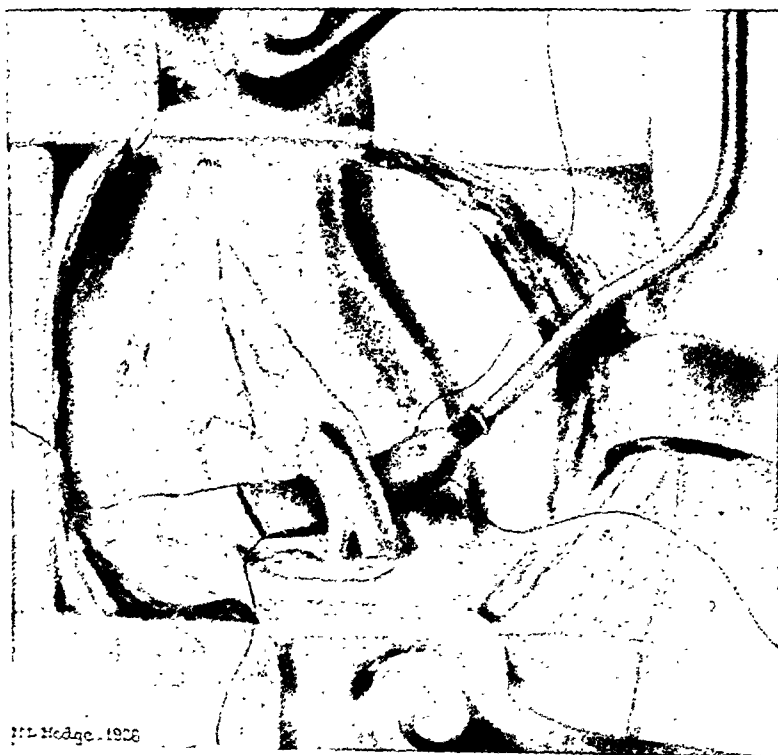
FIG. 2.

FIG. 3.

(Moore and Binger: Blood flow to and from lungs.)

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.*

I. Description of the Strain of Virus Used. Titration and Centrifugation Experiments.

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The findings of the Commission to Study Foot-and-Mouth Disease will be presented in detail in a forthcoming publication by the United States Bureau of Animal Industry. A preliminary paper has already appeared[1] in which were reported comparative studies on vesicular stomatitis and foot-and-mouth disease. In a series of papers to be published in this *Journal*, of which this is the first, the writers will describe their experiments on the physical and chemical properties of the foot-and-mouth disease virus, and their attempts at its cultivation. Of the Commission's personnel, the writers were mainly interested in these particular problems. There was, however, a free interchange of ideas and suggestions, and a hearty cooperation among all the colleagues, so that all publications of the Commission may be regarded as the result of collaboration as a unit.

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* The Commission to Study Foot-and-Mouth Disease was formed by the United States Department of Agriculture, under the direction of Dr. John R. Mohler, Chief of the Bureau of Animal Industry. It comprised Dr. Harry W. Schoening, of the Bureau of Animal Industry, Dr. Jacob Traum, of the Department of Veterinary Science, University of California, and, as Chairman, Dr. Peter K. Olitsky, of The Rockefeller Institute for Medical Research. Dr. Louis Boëz, of the Institute of Hygiene, joined the Commission later as a collaborator.

The writers wish to express their gratitude to the Doyen, Professor Weiss, to Professors Borrel, Vlès, and others, of the Institute and the University of Strasbourg, for their active and whole hearted cooperation.

[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx (n. s. xxiii), 147.

In practically all of the experiments to be reported in the series of papers, we employed, except in a few instances, one strain of foot-and-mouth disease virus. This strain had the advantages of causing lesions promptly and regularly, of acting powerfully, of inducing in practically all instances well marked secondary lesions, and of bringing about relatively few deaths in the experimental animals, thus permitting a long period of observation. The animal used in these studies, unless otherwise stated, was the guinea pig.

Description of Strain of Virus.

On June 18, 1925, through the kindness of a local veterinarian, Dr. Fuchs, a herd of cattle with typical foot-and-mouth disease was placed at our disposal for study. The cattle were kept on a farm about 15 kilometers from Strasbourg. Of twelve animals in the same barn, all in different stages of the disease, one cow was selected which was the last to be infected, and in the 3rd day of illness. She drooled considerably, and had broken aphthæ about the lips, tongue, and buccal mucosa, but no obvious foot lesions, and her temperature was 41°C. It was difficult to aspirate the exudate in the vesicles so that in the end what was brought to the laboratory was an admixture of this cow's saliva and stable refuse. This material was diluted 1:3 with Tyrode solution and then injected, unfiltered, intradermally in the hairless skin of the posterior pads of five guinea pigs. Typical primary vesicles appeared at the sites of injection in 24 to 48 hours in all five. From that time until the investigations were terminated, about a year later, the virus (Strasbourg or "Str." strain) was propagated in the guinea pig for 261 consecutive passages in a pure state, except on two occasions in late passages, when staphylococci were found in the vesicular exudate. These latter were then removed by filtration, without harming the virus. It was found later, by failure of cross-immunity with Vallée's A type, that the Str. strain was probably identical with that of the Vallée "O."

It is to be noted from the effects of transferring the impure virus from the cow to the guinea pig that the virus, when admixed with contaminating microorganisms, could free itself of ordinary bacteria in the tissues of a susceptible animal. Thus a property common to

many filter-passing viruses is shown by the active agent of foot-and-mouth disease. Indeed, after the first series of transfers, no extraordinary care was taken for the cleanliness of the site injected; that this was a proper mode of procedure is gathered from the fact that only twice in 261 passages did the virus become contaminated.

Freedom from Ordinary Bacteria.—Aside from the fact that the virus could purify itself in the guinea pig tissues, deliberate cultivation tests were made when it was first acquired to determine whether there might be any constant bacteria associated with this strain. Repeated cultures were attempted with the blood, with the fluid aspirated from the vesicles (the so called "lymph"), with ground infected pads and the latter filtered—all obtained on the 1st day of illness.

No constant visible microorganisms were revealed by the different methods of culture—the details of which will be described in another paper on experiments on cultivation. Hence this strain was pure, and its effects could not be ascribed to any agent cultivable by ordinary means.

The Disease in the Guinea Pig.—The clinical picture of the experimental disease in the guinea pig corresponded in the main with the comprehensive descriptions given by Waldmann and Pape, by Gins and Krause,[2] and by the members of the British Foot-and-Mouth Disease Research Committee.[3-5] Certain of the more important points are, however, worthy of recapitulation.

Method of Injection.—After a long trial with various methods of injection of the virus, we found that the method of choice, and the one by which practically all guinea pigs were infected, is the intradermal. The procedure consists in running a fine needle in the skin along the length of the hairless pads of the posterior

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[2] For a general historical and descriptive account of the experimental disease in the guinea pig, see Waldmann, O., and Pape, J., *Berl. tierärztl. Woch.*, 1920, xxxvi, 519; and Gins, H. A., and Krause, C., *Ergebn. allg. Path. u. path. Anat.*, 1923-24, xx, pt. 2, 805.

[3] First Progress Report of the Foot-and-Mouth Research Committee, Ministry of Agriculture and Fisheries, London, 1925.

[4] Arkwright, J. A., Burbury, M., Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[5] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

extremities. Attached to this needle is a syringe containing the test fluid, and with pressure on the piston the skin tunnel thus made is filled as the needle is withdrawn. Three or four such injections are made on each posterior pad, and besides, the thin, loose skin along the outer and inner margins of the pad is filled until it distends to a distinct bleb-like formation. If the virus is weak or diluted, an additional inoculation is made subcutaneously in these pads. Inoculations by means of skin tunnels were also found advantageous by the workers of the British Commission.[4,5]

Injection of the virus in sites other than the pad skin was followed by specific lesions, but certainly not as regularly as those occurring after the intradermal inoculation,[6] as the following experiment illustrates.

Protocol 1.—All animals were injected with the same sample of virus taken from a guinea pig in the 1st day of illness. The infected pads of the latter were removed, ground in sand, and suspended in phosphate buffer solution (pH 7.6) to a dilution of 1:25. ⅓ cc. was inoculated in each guinea pig.

(*a*) Intramuscularly in the thigh. Of six animals only two showed the typical aphthæ: in one, in all four pads; in the second, in all pads and in the tongue. Incubation period 48 to 72 hours.

(*b*) Intraperitoneally. Of five animals three exhibited vesicles in all pads and in the mouth. Incubation period 3 to 4 days.

(*c*) Subcutaneous, under the abdominal skin. Of five guinea pigs, two showed aphthæ in all pads and in the tongue, one in two pads, and one in one pad. Incubation period 3 to 6 days.

(*d*) Intracutaneous in abdominal skin. Of five animals, four showed lesions in all pads and in the tongue. Incubation period 3 to 5 days.

In none of these were any lesions noted at the site of the injection.

When these methods of injection are compared with the intradermal one, already described, by which practically all of over 2000 guinea pigs were infected, it will be noted that the latter was more reliable. This test also illustrates the epitheliotropic nature of the infection, for irrespective of the site of injection, the lesions appeared only in the epithelium of the pads or of the tongue or of the mouth. Furthermore, if a proper injection of the virus was given, no natural immunity to foot-and-mouth disease could be found in guinea pigs.

[6] In this place, as in all others in which reference is made to "intradermal" inoculation, we mean to imply the particular method of injection described in the preceding paragraph.

Secondary Lesions.—As already mentioned, the Str. strain induced secondary lesions regularly. These appeared in the pads or mouth or tongue, in sites other than those injected, as early as 36 hours and as late as 5 days, but as a rule from 48 to 72 hours after inoculation. For an unequivocal determination of experimental foot-and-mouth disease in the guinea pig, the presence of these secondary lesions was essential. In doubtful instances, such as when the primary vesicles were obscured by necrosis—a condition encountered frequently in experiments with antiseptics, to be mentioned later—recourse was had to transfer to normal animals for observation of primary and secondary signs before a conclusive diagnosis could be made.

Period of Incubation.—In about 95 per cent of the cases the period of incubation was from 18 to 48 hours. In one instance, the first symptoms were noted after 12 days and in three cases, after 10 days. In the remainder, however, the primary aphthæ appeared from 3 to 6 days after injection. For these reasons, observations were prolonged to 10 to 12 days before an exact definition was obtained; and when necessary, an injection of active material was made in animals which did not react, to determine the presence of immunity. A noteworthy fact is that the period of incubation is shortened and the severity of the disease is increased as the dose or concentration of the virus is augmented.

Time of Greatest Infectivity.—Tests showed that the blood of the guinea pig was active, when withdrawn 24 and 48 hours after intradermal inoculation. On the other hand, if this blood, in a defibrinated condition, was kept at 37°C. *in vitro*, it was active for 24, but not for 48 hours. The period of greatest activity of the virus in lymph aspirated from aphthæ or in ground infected pads was obtained from lesions up to 24 hours in age. From this time to 72 hours there was a gradual diminution in virulence and after 3 days such virus was only exceptionally active. At about this latter time, the active agent, conforming to the tendency of filter-passing viruses in general, invited sometimes invasion by secondary microorganisms, followed by purulent inflammation.

Mortality.—The mortality rate from infection of guinea pigs with the Str. strain was about 1 per cent. As this rate corresponded to the normal death rate among stock animals, it may be stated that

the experimental disease induced by this strain was practically non-lethal.

Transfer to Cattle.—Finally, this strain, after propagation in the guinea pig, could induce, with ease and at will, in cattle and hogs, typical foot-and-mouth disease indistinguishable from the natural affection. The material used for such transfers was derived from guinea pigs either in the early or last passages.

Titration of the Virus.

It was essential to determine quantitatively the activity of the virus, for this estimation was important from the point of view of controlling survival or preservation, as opposed to multiplication, of the virus in subplants of cultivation tests. This information also served as a guide for interpretation of the cataphoresis tests to be reported later, and gave an idea of the relative size of the active agent.

The virus was found to be active in remarkably high dilutions as the following experiments will show:

Protocol 2.—Summary of fifteen experiments. The virus employed for these tests consisted of the aspirated lymph from 24 hour old vesicles. The diluent consisted of phosphate buffer solution at pH = 7.5.[7] Dilutions were made with pipettes. The diluted virus was injected by the intradermal method, already described, in both posterior pads of guinea pigs.

Of fifteen tests, the virus was found active in a dilution of 1:10,000,000, twice; 1:2,000,000, thrice; and 1:1,000,000, twice. In the foregoing trials, these were the final dilutions. In four additional tests where dilutions were made higher than that of the active range, in one case 1:4,000,000 was positive, but 1:8 to 32,000,000, negative; in another, 1:2,000,000 was active, but not 1:4 to 80,000,000; in a third, 1:1,000,000 was positive, but 1:2,000,000 negative; in the last case, 1:400,000 was active, but not 1:1,000,000.

All these experiments were made with the virus cleared of particles by filtration through filter paper. In the next four tests filtrations were made through Berkefeld V candles. Activity was shown in all four instances in a dilution of 1:2,000,000, the highest made.

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[7] Phosphate buffer, as mentioned here and elsewhere in these articles, is made by adding 2.5 gm. potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

From these titrations it is to be noted that the virus diluted to 10 million times its volume may still be effective. This fact leads to several considerations, the first of which is with reference to the probable size of the active agent. Since the material is not solid with virus, but contains the diluent and various products usually found in an inflammatory exudate, the active agent is only a part of this quantity. Hence the agent should be quite minute. That the size of the virus is less than $100 \, m\mu$ has also been confirmed in the study on "molecular" filtration, to be described in another paper.

Secondly, great care should be exercised in measuring or transferring virus in glass receptacles in tests for comparative activity, such as the determination of the killing effect of antiseptics, the relative value of different media in cultivation tests, etc. Spattering of the virus along the glass walls may give rise to infections which may have no bearing on the problem in hand. We have therefore substituted for test-tubes, whenever practical, chemical mixing glasses or conical beakers.

A third point is that in our experiences with cultivation of the virus, the third subplant showed activity in five different media. In this third subplant the virus was diluted by our method of seeding about 1:1,000,000. But in the fourth subplant the active agent was diluted 1:15,000,000. In this latter, however, the virus was without action. Apart from this, we had other and ample evidence to show that these media were not suitable for growth. Hence the conclusion derived from such experiments is that preservation or survival, but not multiplication is involved. Therefore, the work of Titze[8] and of others who maintained virulence only in the first few "subcultures" should be interpreted as probably indicating a mere dilution of the active agent.

Another consideration is that since one sample of the virus is active in a dilution of 1:400,000, and another in a dilution of 1:10,000,000, a factor of difference in activity between one specimen and another is 25. The investigators of the British Committee[3] have found a still greater factor to exist; one sample was inactive at 1:5000, and another active at 1:500,000. Hence, in comparative tests, such as

[8] Titze, C., Z. Infektionskrankh. Haustiere, 1924, xxvi, 107.

in the study of the survival of the virus in different media, trials should be made with the same specimen of virus.

Finally, the severity of the disease and the incubation period depended on the concentration of the virus: the more concentrated the virus, the more severe was the infection and the shorter the period of incubation. For example, with one sample of a 24 hour aspirated lymph virus, a dilution of 1:1000 induced the experimental disease of severe type in 24 hours in all of five guinea pigs; of 1:200,000, after 3 days, in one of two animals; of 1:2,000,000, after 4 days, mildly in one of two animals; and of 1:4,000,000, after 9 days, one vesicle was noted on the tongue of one guinea pig, of two inoculated. Higher dilutions were negative.

The rate and energy of action, therefore, are proportional to the concentration of the active agent.

Centrifugation.

Centrifugation tests were made with two objects in view: (1) to determine the centrifugability of the virus, and (2) if the virus were not centrifugable, to attempt to free, by this process, the supernatant fluid from a hypothetical body which may be the inhibiting factor in cultures, for example such bodies as fragments of tissue or cells.

As for the first object, it was determined by repeated tests that with the active guinea pig blood, aspirated lymph, or suspensions of ground infected pad tissue, centrifugation at 2500 to 3000 revolutions per minute for 2 hours did not cause the deposition of the virus: the lowest portion of the specimen was as active as the topmost layer. Thus we have confirmed the prior findings of others, notably the workers of the British Committee,[3–5] Abe,[9] and Gins.[10] However, we could not confirm thereby Frosch and Dahmen's[11] first step in cultivation, namely a concentration or sedimentation of the virus by centrifugation at 3000 revolutions per minute for $\frac{1}{2}$ to $1\frac{1}{2}$ hours.

The inability to sediment the virus may indicate that the active agent is very minute, but not necessarily that it is of the nature of a

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[9] Abe, T., Z. Infektionskrankh. Hausticre, 1925, xxviii, 111.

[10] Gins, H. A., Berl. tierärztl. Woch., 1924, xl, 661.

[11] Frosch, P., and Dahmen, H., Berl. tierärztl. Woch., 1924, xl, 185, 273, 341; Arch wissensch. u. prakt. Thierheilk., 1924, li, 99.

contagium vivum fluidum. Duclaux[12] has shown that centrifugation is not an adequate means for deposition of minute particles. For theoretically with a centrifugal force 40,000 times as great as the gravity force of a particle (such as is obtained in large centrifuges) a particle measuring 10 mμ will settle only 1 cm. in 4 hours. But practically, in addition to this slow deposition due to size, the whirling current and that due to convection, since all machines develop a certain amount of heat, operate to prevent deposition. In the example cited the variations due to these currents should be multiplied by 40,000 to obtain the actual reading of the forces preventing sedimentation (Duclaux).

In respect to the removal of a hypothetical inhibiting body by centrifugation, our repeated experiences showed that the active guinea pig blood kept at 37°C. contained no virus either in the sediment or supernatant fluid, when tested 3 days after centrifugation. But at room temperature similar ground pad virus suspended in phosphate buffer at pH $\approx$ 7.5, remained alive for 9 days in the sediment and for 12 days in the supernatant fluid. With aspirated lymph under the same conditions, however, both the sediment and supernatant fluid were active for at least 14 days. No further tests were made in this instance.

To complete these experiments, three additional trials were made with the sediment after showing inactivity by standing for 4 days at room temperature. This sediment was added to either fresh, or old, active supernatant fluids, and the mixture kept for from 18 hours to 3 days at 20°C. But no inhibition of the virus in the supernatant fluid was observable.

We conclude from these experiments that the hypothetical virucidal bodies cannot be deposed from suspensions of ground infected pad tissues, or of aspirated lymph, by centrifugation.

At this point, brief reference may be made to attempts to remove substances which inhibit the viability or multiplication of the virus in artificial media, by precipitation of the globulins, or inactivation of complement. For this purpose carbon dioxide was employed,

[12] Duclaux, J., Les colloïdes, Paris, 2nd edition, 1925, 84 ff.

and prolonged passage of the gas was allowed through material containing the active agent.   But these tests likewise ended in failure.

SUMMARY AND CONCLUSIONS.

A strain of foot-and-mouth disease virus was recovered from a cow at the height of the disease, and was propagated through at least 261 passages in the guinea pig.   Considerably over 2000 animals proved susceptible to the virus, and the virus could be transferred at will back to cattle and hogs, and then again returned to guinea pigs.   No natural immunity was discovered in guinea pigs.   Secondary lesions were easily and regularly induced, thus making this strain particularly favorable to experimental purposes.   In general, the guinea pig may therefore be regarded as the animal of choice for laboratory studies.

The guinea pig could be infected by different methods of injection in different sites, but constant and regular production of primary and secondary lesions—or generalization of the disease—followed intradermal "tunneling" in the manner described, combined with subcutaneous inoculation of the posterior hairless pads of full grown animals.   As we have indicated, the virus was peculiarly epitheliotropic, which in turn gives support to the opinion that its portal of entry may be limited.

The active agent could purify itself of chance concomitant bacteria in the first passages, in a susceptible animal—a character possessed by filter-passing viruses in general.

The virus was active in dilutions of 1:10,000,000.   This shows not only the minuteness of the active agent, but also the necessity for a change of technique from that employed with larger sized infectious agents.   Apart from this, the dilution factor is important in interpreting mere preservation of the virus rather than multiplication, when only early successive subplants in culture experiments are positive.   Furthermore, some samples of virus were not so active—a factor of twenty-five existed between the weakest and strongest samples among fifteen titrated.   This indicates that comparative tests, as, for example, of survival in different media, should be made with the same specimen.   In any case, the rate and energy of action of the virus were proportional to its concentration, thus differing from the behavior of certain enzymes.

The incitant is not sedimented by centrifugation. Non-centrifug-ability, a property of some other filter-passing, infectious agents, is not an indication of the fluid character of the virus, as we have already explained. In view of the evidence presented and other tests to be reported later, failure of deposition is related to the minute size of the incitant. The method of centrifugation has also failed to remove "virucidal bodies" in the meaning of Frosch and Dahmen.

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STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

II. CATAPHORESIS AND FILTRATION.

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In the first[1] of this series of papers we described the strain of foot-and-mouth disease virus employed in our studies. We have also reported therein that guinea pigs were susceptible to the active agent in dilutions of 1:10,000,000, and that the virus was non-centrifugable. The indications pointed to a minute size of the incitant.

In the experiments to be presented in this article on cataphoresis and filtration, the findings regarding the minuteness of the incitant are confirmed and, furthermore, a relative delimitation of its size will be demonstrated. In addition, the electric charge carried by the virus will be revealed and phenomena dependent on the charge and on certain filtration reactions will be discussed.

Cataphoresis.

The electric charge carried by the active agent, as well as its isoelectric range, was a criterion by which different filtration tests could be interpreted. Its determination was useful not only to explain the remarkable resistance of the virus to certain chemicals to be described elsewhere, but also to compare the charge with that characterizing certain known microorganisms.

The main difficulty of cataphoresis experiments with this virus was the absence of a recognizable or visible particulate body. But this was overcome by adapting to our purposes the Vlès[2] apparatus.

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[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

[2] We wish to express our gratitude to Professor Fred Vlès of the University of Strasbourg for his cordial cooperation and his invaluable assistance, not only in these experiments, but also in others on filtration.

Moreover, guinea pigs could be regularly infected with even minute quantities of the active agent, so that its attraction to one or another pole could be determined by the biological test.

The apparatus[3] employed consists essentially of horizontal U-tubes, containing in the curved arms a stoppered ampulla, and in each long arm a stop-cock. These are connected to communicating cups by curved syphons which are joined to each other by a transverse tube carrying also a stop-cock. The latter allows a prior establishment of a hydrostatic equilibrium. The orifices of the curved syphons are plugged with moist cotton to prevent hydraulic disturbances. The tubes, fixed to a board, are placed in series of four (because four determinations at different hydrogen ion concentrations were made at one time). At either end of the series, union with the house current is made through a solution of cupric sulfate, and in the connecting cups through physiological saline solution (0.9 per cent at pH = 8). In the syphons of each tube of the series is placed the phosphate buffer[4] at the hydrogen ion concentration at which the virus is tested. In the ampullæ is added the virus, diluted with phosphate buffer at different pH readings, thus filling completely the respective U-tubes to the stop-cocks.

In the following experiments the difference of potential at the limits of the series in an open circuit was 220 volts. A milliamperemeter was then placed in the circuit, which registered from 0.8 to 4.7 ma. for the four tubes, and the fall of potential for each tube was from 20 to 50 volts. The time of cataphoresis lasted from 20 to 70 minutes. We have found that the best result was obtained when the virus was filtered and diluted 1:1000 in phosphate buffer, at a milliamperage of 0.8, with a fall of potential of 20 to 30 volts and cataphoresis for 70 minutes.

This experiment, one of a series of ten, is illustrative:

Protocol 1.—24 hour aspirated lymph virus was diluted 1:1000 with phosphate buffer at pH = 7 5, and filtered through Berkefeld V candles. This material, on injection intradermally in the pads of guinea pigs, showed activity in each of graded dilutions from 1:2000 to 1:200,000.

The substance was then separated into four portions and each was adjusted to different pH readings by the colorimetric method. At the same time phosphate

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[3] Vlès, F., and de Coulon, A., *Arch. physiq. biol.*, 1924, iv, 43.

[4] Phosphate buffer is made by adding 2.5 gm. potassium acid phosphate (KH_2PO_4) to a liter of distilled water. This is then adjusted to the desired pH by means of potassium hydroxide. It is of utmost importance to readjust the material just before use, for sterilization renders it acid.

buffer adjusted to similar degrees was prepared. At the termination of the cataphoresis experiment the following electrometric pH measures were obtained:

Tube	Buffer	Virus	Average buffer and virus
1	6.63	6.84	6.73
2	7.68	7.62	7.65
3	8.00	7.90	7.95
4	8.17	8.05	8.11

The cataphoresis was continued for 70 minutes with a fall in potential of 20 to 30 volts with a milliamperage of 0.8.

Guinea pigs were then injected intradermally in the pads with the material removed from the negative and positive poles of each of four differently adjusted specimens with these results:

pH = 6.73

    Negative pole: 2 guinea pigs; both positive after 48 hours.

    Positive pole: 2 guinea pigs; both negative.

pH = 7.65

    Negative pole, 1:2000: 2 guinea pigs; both positive after 48 hours.

                    1:20,000: 2 guinea pigs; both positive after 48 hours.

    Positive pole, 1:2000: 2 guinea pigs; both negative.

                    1:20,000: 2 guinea pigs; both negative.

pH = 7.95

    Negative pole, 1:2000: 2 guinea pigs; both positive after 48 hours.

                    1:20,000: 2 guinea pigs; both positive after 48 hours.

    Positive pole, 1:2000: 2 guinea pigs; both negative.

                    1:20,000: 2 guinea pigs; both negative.

pH = 8.11

    Negative pole: 2 guinea pigs; both negative.

    Positive pole: 2 guinea pigs; both positive after 48 hours.

Results of other tests at pH readings different from those mentioned showed:

pH = 6.6

    Negative pole, 1:500: 2 guinea pigs; both positive after 48 hours.

    Positive pole, 1:500: 2 guinea pigs; both negative.

pH = 7.0

    Negative pole, 1:500: 2 guinea pigs; both positive after 48 hours.

    Positive pole, 1:500: 2 guinea pigs; both negative.

pH = 7.4

    Negative pole, 1:500 to 1:100,000: in all dilutions, all guinea pigs positive after 48 hours.

    Positive pole, 1:500 to 1:100,000: in all dilutions, all guinea pigs negative.

pH = 7.5

    Negative pole,   1:250 to 1:250,000: in all dilutions, all guinea pigs positive
                 after 2 to 4 days.

    Positive pole,   1:250 to 1:250,000: in all dilutions, all guinea pigs negative.

pH = 8.0

    Negative pole,   1:150 to 1:30,000: in all dilutions, all guinea pigs positive
                 after 48 to 72 hours.

    Positive pole,   1:150 to 1:30,000: in all dilutions, all guinea pigs positive
                 after 24 to 48 hours.

pH = 8.5

    Negative pole,   1:500: 2 guinea pigs; both negative.

    Positive pole,   1:500: 2 guinea pigs; both positive after 48 hours.

It appears, therefore, that the foot-and-mouth disease virus is capable of wandering in an electric field and then, if the active agent is at a hydrogen ion concentration below pH = 8, the direction of motion is toward the negative pole (electropositive); if above pH = 8, toward the positive pole (electronegative).   At about pH = 8, there is a tendency to move in either direction (isoelectric range).   Hence it may be stated that under ordinary conditions, the active agent has an electropositive charge, and that its isoelectric range is at pH = about 8.   It should be remembered that, in view of the absence of a visible particulate body, and the slight changes in hydrogen ion concentration during cataphoresis, this figure is relative; hence the term "range" is employed rather than the word "point."

It was noted that, as a rule, there was clouding at the pole opposite to that to which the virus was attracted.   For example, if the fluid at the positive pole appeared hazy, and at the negative limpid, then the material at the negative pole contained the active agent.   Whether the force of the electric current is sufficient to tear the virus from the electronegative protein particles to which it may be attached,[5] we are unable to say, but these findings are suggestive.   The possible objection that the virus wanders along with protein does not hold, for the virus moved in a different direction from the ordinarily electronegative proteins.

The fact that the virus has an unusually high isoelectric range and

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[5] In another section will be described the adsorption of the virus to large protein coagula.

is, under ordinary conditions, electropositive does not speak against its possible protein or living nature. For, although bacteria are generally electronegative in charge, spirochetes, as a genus, carry electropositive charges (with the exception of *Treponema pallidum*); so do trypanosomes, such as the species of *brucei, equiperdum, gambiense*, and *rhodesiense*. Among protein substances, fibrin and gliadin (at pH = 9.3) are known to possess a high isoelectric point. The outstanding fact is that the virus, in respect to its charge, is different from ordinary bacteria.

The determination of the charge, however, was of greatest importance in interpreting the results of ordinary and "molecular" filtration experiments, and therefore gave indirectly some indication of the relative dimensions of the causal agent.

Filtration.

The filters employed in the following experiments comprised the Seitz' asbestos discs, Berkefeld V and N candles, Chamberland's L 1, L 2, L 3, L 5 (F), L 7 (B), L 9, L 11, and L 13 bougies, various thicknesses of collodion membranes, and, finally, Bechhold's ultra-filter prepared with different percentages of acetic collodion.

It is important to note that the foot-and-mouth disease virus, as employed in the following tests, may contain the incitant in a free state, as well as adsorbed to particles. That the virus can be free and that there is a lack of complete adsorption to a number of different substances has been observed by several investigators (Dahmen, Gins, Bedson, Burbury and Maitland, and Stockman and Minett). Furthermore, there is practically no loss in activity of the virus after filtration through coarse and fine filters. In addition, filtrations proceed in a definite, regular manner. For these reasons, and from the results of cataphoresis experiments showing a separation of the virus from protein particles, one cannot regard the whole of the incitant as adsorbed to minute particles of the same size.

Experience with the Seitz' filter showed that the virus filtered easily through one disc of asbestos. We have not employed this method for study of filtration phenomena, but merely to purify the

virus from contaminating microorganisms. For this purpose, the Seitz' filter is quite effective.[6]

In regard to the other methods of filtration, a general principle was involved in all cases, namely, all the filters carry an electronegative charge. As the virus has an electropositive charge, considerable adsorption occurs during the passage of the active agent through the candles, bougies, or membranes. In some filters in which the pore size is minutely small, and the charge strongly negative, complete adsorption takes place with the consequence that the filtrates contain no virus.

Filtration through Berkefeld V and N Candles.—The material for these tests consisted of aspirated lymph, or of ground infected pads suspended in phosphate buffer at pH = 7.5 or 7.6. In any event, preliminary filtration through one layer of filter paper was resorted to. This was done to prevent clogging of the small candles with particles of tissue, fibrin, or cells.

As tests of the charge and relative pore size of these filters, there were passed through them suspensions containing particles of either charge and of different sizes. Electropositive micellæ, such as methylene blue, basic fuchsin, and night blue regularly failed to pass, but electronegative particles, such as acid eosin and Prussian blue, readily traversed the candles. Hence these filters carried an electronegative charge.

It was therefore necessary, in determining the size of particles capable of passing through the V and N filters, to eliminate adsorption due to filtering oppositely charged substances. Accordingly, collargol, the mean diameter of its micellæ being 20 mμ and colloidal arsenic trisulfide, 100 mμ, were tested. Both passed rapidly through V, and definitely, but less speedily, through N candles. Both substances have an electronegative charge. Hence these Berkefeld filters, with the adsorption action counteracted, allow the passage of particles of at least 100 mμ in diameter. This agrees in part with the determination of the size of the intergranular spaces by Bechhold's formula,

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[6] For more extensive studies on this type of filter, the reports of the investigators of the British Committee should be consulted. References are cited in the first paper of this series.

as given by Mudd.[7]   The latter found the diameter of the V type to be 0.38 μ (380 mμ), and of the N type, 0.45 μ (450 mμ).

In respect to the virus of foot-and-mouth disease, a standard substance for comparison should be one with a positive charge, and of a definite known size.   For this purpose, pure, crystallized hemoglobin in solution, and therefore molecular, was employed.   Its isoelectric range is at pH $= 6.75$, and the size of its molecule, as computed by Bechhold,[8] is 3.6 mμ in diameter.   Suspended in phosphate buffer (employed also in suspensions of the virus) at pH $= 6$, hemoglobin is electropositive, and at pH $= 7.5$, electronegative.   In the former condition this substance passes through V and N candles; in an electronegative condition, the passage is more free and completely unobstructed.   Since the virus with an electropositive charge also passes regularly through both these filters, showing at times activity in at least a 1:2,000,000 dilution of the filtrate, the conclusion derived from these observations is that the active agent is at least larger than the molecule of hemoglobin.   This has been confirmed by our experiences with filtrations through Chamberland bougies.

Filtration through Chamberland Bougies.—The Chamberland bougies of all types are similar to the Berkefeld candles in that they are charged electronegatively.   For, electropositive particles, such as methylene blue, basic fuchsin, and night blue dye do not pass through their walls, but the electronegative micellæ of acid eosin, Prussian blue, collargol, and colloidal arsenic trisulfide do.   In regard to hemoglobin, at pH $= 6$, with an electropositive charge, this substance passes these filters only slightly—the first few drops of filtrate are clear but after a time traces of hemoglobin are revealed by spectroscopic examinations.   At pH $= 7.5$, with an electronegative charge, hemoglobin traverses freely all the types of Chamberland filters.   In other words, as an electropositively charged molecule, hemoglobin first satisfies the avidity of the electronegative material of the bougie and later its minute size enables it to pass through the barrier.

A similar experience was obtained with the electropositive virus. With bougies of graded series to L 5, the active agent traversed their

[7] Mudd, S., *J. Bact.*, 1923, viii, 459.

[8] Bechhold, H., *Z. physik. Chem.*, 1908, lxiv, 328.

walls. But with more dense types, the passage became more difficult, until with the L 11 type,[9] the filtrates were free from virus. For example, five trials with five different new L 7 type bougies yielded in only one case a positive filtrate; three filtrations through three different, new L 9 type bougies, resulted in one positive; and seven tests with seven different L 11 type bougies, six of which were new, the seventh having been used more than once, failed in all instances. In these experiments the virus was suspended in phosphate buffer at pH = 7.5 (electropositive). Diluted in similar material at pH = 8.5, the virus, then in an electronegative state, passed freely through these bougies.

The pore size of the L 11 type of Chamberland filter has been determined after Bechhold's[8] air pressure, revised method, as 110 to 150 mμ. It appears, therefore, that the foot-and-mouth disease virus can, by elimination of adsorption due to opposite electric charges, traverse these small spaces. On the other hand, if adsorption due to opposite charges is unhindered, the virus fails to pass. But, in the latter relation, hemoglobin under similar conditions can traverse this filter. Hence the active agent is larger than the hemoglobin molecule of 3.6 mμ diameter. Experiments with filtration through collodion membranes, to be described immediately, confirm this relative measurement.

Filtration through Collodion Membranes.—For a determination which might give a measurement of the virus between narrower limits, and for a more extended study of the filtration phenomena, a still tighter filter was employed, namely, the collodion membrane.

Collodion membranes are negatively charged. Indeed, the individual particles of pure collodion remain so charged, or become even more strongly negative, in the presence of either acid or alkali, as measured by the cataphoretic potential difference between these particles and water (Loeb[10]).

The collodion membranes, usually in the form of small sacs, were

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[9] Several trials with a number of the L 13 type bougie, supposed to be the tightest of all of the series, resulted in irregular filtrations of titrating reagents. Hence work with this type was discontinued.

[10] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 79 ff.

prepared with French Codex Collodion (5 per cent). In all 68 sacs were made with different proportions and conditions of collodion, so that eighteen different experiments were performed. The concentration of colloidion varied from 5 per cent to 12 per cent (the 12 per cent corresponding to 15 seconds reading on the apparatus devised by Gates,[11] for measuring its relative viscosity). From one to three layers were used, and dipping in 95 per cent alcohol was resorted to in some instances to increase the permeability of the sacs. Negative pressure from 10 to 25 cm. of mercury was applied. The virus comprised 24 hour aspirated lymph, diluted 1:50 or 1:100 in phosphate buffer at pH = 7.5 or 7.6 to pH = 8.5 which in all tests, as measured by control inoculations in guinea pigs, was quite active. Six experiments were also made with sacs prepared after the manner of Levaditi, Nicolau, and Galloway,[12] who used three layers of 5 per cent collodion (Codex), without alcohol immersion.

To summarize the results of these experiments: We found that the collodion membranes prepared in the manner stated are not suitable for the filtration of the virus of foot-and-mouth disease. For more than half of the number of membranes showed macroscopic holes before put to test. In the remaining ones, we could not convince ourselves that microscopic holes were absent. In the absence of standardization of the membrane, it was necessary that each sac be tested separately by titration with known substances of definite charge and size. Otherwise the results were valueless. Membranes which we thought hole-proof and at the same time made so as to withstand a negative pressure of from 10 to 25 cm. mercury (without this negative pressure, filtration was impossible), failed, as a rule, to allow passage of methylene blue, eosin, collargol, hemoglobin, etc.—the materials employed for titration of the Berkefeld V and N and all types of Chamberland filters. They did allow the passage of peroxidase (which we found to be more easily filterable than blue litmus), of blue litmus, the size of which is computed by Bechhold[8] to be 1.8 mμ in diameter, and of crystalloids. The crystalloids which traversed the membranes were, in order of molecular weights from

[11] Gates, F. L., J. Exp. Med., 1921, xxxiii, 25.
[12] Levaditi, C., Nicolau, S., and Galloway, I.-A., Compt. rend. Acad., 1926, clxxxii, 247.

strychnine, molecular weight, 397; acid quinine sulfate, to basic quinine sulfate, molecular weight, 890.[13]   The virus, on the other hand, failed to pass through these membranes.

It may therefore be concluded that collodion membranes of the types mentioned are permeable to colloids containing particles in size of the order of blue litmus, and to crystalloids, but not to the virus carrying positive or negative charges.   Furthermore, in the absence of standardized membranes, they are unsuitable for titration experiments.   We have, however, confirmed the observation of Stockman and Minett[14] that the virus does not traverse two layers of 5 per cent collodion.   One conclusion may be derived from these experiments: the active agent is not comparable to crystalloids in respect to filtration through collodion membranes.

It was then found necessary to seek a more permeable membrane, by which one could delimit the size of the virus more precisely and, at the same time, be free from the variables in the methods already mentioned.   For these purposes we found the Bechhold ultrafilter membranes quite satisfactory.

Filtration through the Bechhold Ultrafilter Membranes.[15]—The prepared Bechhold discs are made by impregnating in a vacuum Schleicher and Schüll filter paper with different concentrations, from $1\frac{1}{2}$ to $7\frac{1}{2}$ per cent, of acetic collodion.   By this method unevenness of the layer and microscopic holes are eliminated; the membrane is strong enough to withstand the necessary negative pressure and a concentration of as little as $1\frac{1}{2}$ per cent of the collodion can be used with, as a rule, unequivocal results.

We adapted these discs to our purposes by cutting each membrane to a circumference corresponding to that of the lower part of a Seitz (Manteufel,[16] E. K. model) filter.   The membrane was placed over the wire gauze support and a smooth, well fitting, firm, rubber washer

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[13] Strychnine was tested for its presence in the filtrates by inoculation of frogs; quinine, by opalescence, in a strong beam of light, after acidulation with sulfuric acid.

[14] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

[15] The membranes are manufactured by Schleicher and Schüll, Düren, Rhineland.

[16] Manteufel, P., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 259.

was superposed. The cup end was then tightly screwed in and the mechanism was tested for leakage at the negative pressure employed in each test.

The Bechhold disc is electronegative. To eliminate the adsorption of substances oppositely charged, both the virus and the test materials for titration were employed in a condition of the same, that is, negative charge. Hence, for virus, we prepared 24 hour old lymph as-

TABLE I.

Filtration through Bechhold's Ultrafilter Membranes.

Substance	Charge	Size of particle	7½ per cent		6 per cent		4½ per cent		3 per cent					1½ per cent				
			Number of membranes employed	Results	Number of membranes employed	Results	Number of membranes employed	Results	Number of membranes employed	Results in each case. Membrane No.				Number of membranes employed	Results in each case. Membrane No.			
										1	2	3	4		1	2	3	4
Virus	Neg.	X	4	−	4	−	4	−	4	+	−	−	−	4	+	+	+	+
Blue litmus	Neg.	1.8mμ	4	+	4	+	4	+	4	+	+	+	+	4	+	+	+	+
Hemoglobin 1 per cent	Neg.	3.6mμ	4	−	4	−	4	−	4	+	+	+	−	4	+	+	+	+
Collargol	Neg.	20mμ	4	−	4	−	4	−	4	+	−	−	−	4	+	+	+	+
Colloidal arsenic trisulfide	Neg.	100mμ	4	−	4	−	4	−	4	−	−	−	−	4	+	−	−	−

− indicates the non-filterability and + the filterability of the substance tested. With the 4½ to 7½ per cent collodion membranes, the results were uniform. With the 1½ and 3 per cent membranes, there were slight variations, so that the tests with each are tabulated.

pirated from fresh, unbroken vesicles, diluted to 1:100 in phosphate buffer at pH = 8.5. The substances for titration consisted of blue litmus and hemoglobin, each suspended in the phosphate buffer at the same hydrogen ion concentration. The collargol and the colloidal arsenic trisulfide, both electronegative, were prepared fresh after the directions of Ostwald.[17] As a test for the charge of the membrane

[17] Ostwald, W., Manipulations de chemie colloidale; translated by Veillinger, E., from the 4th German edition, Paris, 1924.

and its ability to adsorb an oppositely charged substance, a suspension of electropositive night blue dye (*bleu de nuit*) was used. Complete adsorption of this dye occurred in every case.

In Table I are summarized the experiments made with membranes of different permeability, that is, of different thicknesses, containing from $1\frac{1}{2}$ to $7\frac{1}{2}$ per cent acetic collodion. The virus under electronegative conditions is compared with the electronegative test substances, blue litmus, hemoglobin, collargol, and colloidal arsenic trisulfide. These varied in size from 1.8 mμ to 100 mμ in diameter. The negative pressure required for each type of membrane differed with the thickness of the layer. For example, with the $7\frac{1}{2}$ per cent membrane, -30 cm. mercury pressure was employed; with the 6 per cent, -10 cm.; with the $4\frac{1}{2}$ per cent, from -5 to -10 cm.; with the 3 per cent, -5 cm.; with the $1\frac{1}{2}$ per cent, -5 cm. In all cases the filtrates were tested for the presence of virus by intradermal inoculation of the hairless pads of guinea pigs; and for the titrating substances by colorimetric, spectroscopic, and chemical tests. It may be stated here that the virus taken from the apparatus, before filtration and at pH $= 8.5$, was injected intradermally in three to four guinea pigs in each instance. After 24 hours all animals showed the typical experimental disease.[18]

An examination of the tabulated, summarized results reveals that, in general, there are only a few irregularities in filtration through the single layer Bechhold membranes—indeed, fewer than were expected. But in view of the fact that each membrane was titrated and its porosity delimited, the results of "molecular" filtrations appear to be conclusive. Furthermore, the selective passage of the titrating substances which we have employed is identical to that reported by Bechhold.[8]

Only the minute particles of blue litmus could traverse the $4\frac{1}{2}$ to $7\frac{1}{2}$ per cent membranes and then quite regularly. The virus uniformly failed to pass. The virus did, however, traverse one of four membranes of the 3 per cent type. In that instance collargol also passed. In the three others of this series, two allowed the passage of hemo-

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[18] In these, as in all filtrations, only from 5 to 10 cc. of material was filtered, and the time regulated. Results therefore cannot be ascribed to employment of large quantities, filtered over a long period.

globin and one the blue litmus only. On the other hand, the virus filtered uniformly through all membranes of the $1\frac{1}{2}$ per cent type. In these the porosity was of such an order of magnitude that in all collargol traversed the membranes as well, and in one case, the colloidal arsenic trisulfide.

One may conclude, therefore, that the order of magnitude of the active agent of foot-and-mouth disease, after elimination of interference by adsorption due to unlike charge, is, in relative terms, correspondingly between that of collargol and of colloidal arsenic trisulfide particles. In other words, the size of the virus is relatively between 20 and 100 mμ.[19]

These tests have not only served to delimit the size of the active agent, but also serve to refute the notion that the virus is a fluid or a *contagium vivum fluidum*, after the meaning of Beijerinck,[20] of which more will be said later. Furthermore, the same comparative ease with which the virus and collargol particles filtered through the $1\frac{1}{2}$ per cent membranes and the difficulty with which colloidal arsenic trisulfide traversed similar discs indicate that the size of the active agent may be nearer the minimal reading than the maximal. The minuteness of size may explain the invisibility of the virus; the difficulty of its artificial cultivation and its resistance to alcohol and other chemicals, to be described in another paper; its non-centrifugability; and its activity in very high dilutions. For particles of these relative dimensions are governed by different physical laws than those influencing larger structures.[21] Finally, the question arises as to whether these measurements preclude the notion that the active agent is living. In the absence of a standard for comparison, we may compare the virus to bacteria in respect to their chief function, the enzymatic, and their composition, which is mainly protein. We have determined (*vide supra*) that peroxidase can pass freely through spaces which do not permit the passage of litmus, the particles of which are

[19] These tests supply a basis of only a relative calculation. Hence it was decided, especially in view of the definite limitation of time for experimental work in Europe, to forego further trials in an attempt to narrow the range of size to closer approximations.

[20] Beijerinck, M. W., *Centr. Bakt., 2. Abt.*, 1899, v, 27.

[21] Vlès, F., *Rev. scient.*, 1921, lix, 294.

estimated at 1.8 mμ in size (Bechhold). On the other hand, assuming the virus to be spherical and of a diameter of 50 mμ, it should be more than 2500 times the volume ($V = \frac{4}{3}\pi r^3$) of the protein molecule hemoglobin. Furthermore, Vlès[22] has calculated that spherical bodies of the order of magnitude of 30 mμ may contain 360 protein molecules, and those of 90 mμ, 9000.

<div style="text-align:center">SUMMARY AND CONCLUSIONS.</div>

Cataphoresis experiments show that the virus of foot-and-mouth disease carries, under ordinary conditions, an electropositive charge. Its isoelectric range is at the high point of pH = about 8. Although cultivable bacteria are, as a rule, electronegative, yet certain protozoa, such as trypanosomes and spirochetes are also electropositive. In respect to charge, then, the virus is different from ordinary bacteria, but there is nothing in this finding to indicate an inanimate character of the incitant. A knowledge of the charge, however, aids in the interpretation of certain filtration phenomena, and indirectly in delimiting the size of the virus. In addition, it serves to explain its remarkable resistance to certain chemicals—a subject to be dealt with in the next paper of this series. Finally, cataphoresis indicates the possible separation of the virus from protein particles.

Filtration experiments were made with different types of filters: Seitz, Berkefeld V and N, and Chamberland, of practically all sizes, collodion membranes, and Bechhold's ultrafilter. The results confirm the electropositive charge of the virus, as well as the minuteness of its size. Filtration was effected through the Seitz, Berkefeld, and Chamberland filters. In regard to the latter, the active agent passed through the L 11 only when its charge was shifted to negative: under ordinary conditions, carrying an electropositive charge, it failed to traverse this more dense wall, and was completely adsorbed in the oppositely charged barrier. Filtrations through electronegative collodion membranes, prepared in different ways and of varying thicknesses, resulted, as a rule, in failure, unless the thinnest and most permeable membranes were employed. But in these, the complication of microscopic holes was to be considered. Hence this method

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[22] Personal communication.

was regarded as impracticable. Success, however, was obtained with Bechhold's ultrafilter membranes of the most permeable type, and with these it was possible to measure relatively, by a system of "molecular" filtrations, the size of the incitant. This was found to be, in relation to other particles of like charge, between 20 and 100 mμ in diameter.

The filtration phenomena of the foot-and-mouth disease virus can be accounted for on the basis of the minute size of the particles of the incitant carrying an electropositive charge, and no evidence can be deduced therefrom that the virus is of a fluid character. For the relative size of the particles is constant and the invariability of the limits of measurement contradicts the notion that the incitant may be a "solute" varying in dimensions in different "solvents."

STUDIES ON COMPLEMENT FIXATION IN TUBERCULOSIS. III.

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In the course of the work recorded in two previous papers (1, 2) on this subject, increasing interest has developed in a substance extractable from dried tubercle bacilli by hot alcohol, which serves as an antigen in the complement fixation reaction. Much work on the antigenic constituents of the tubercle bacillus and their chemical characterization has appeared recently. Wadsworth, Maltaner and Maltaner (3) have increased our knowledge of the lipoidal constituents and have used them in practical diagnostic work. The experience of these authors is in entire accord with my own in that, in so far as could be determined with the immune serum available to them, bacillary products when subjected to *prolonged* extraction with lipoid solvents, are deprived of residual antigenic activity. Others, particularly Dienes and Schoenheit (4, 5), while recognizing the presence and importance of the constituents extractable by the lipoid solvents, laid stress on the fact that with their sera and antigenic preparations, antigens residing with the protein fractions are also demonstrable and that some sera react with the lipoidal antigens, others with the protein. In no case, however, is it clear that their protein preparations are free from substances extractable with hot alcohol.

The work of Zinsser and Mueller (6) and of Petroff (7) emphasized the importance of "residue" after precipitation of protein-containing extracts with heat and acetic acid. These observations brought the work on the tubercle bacillus into closest conjunction with the work of Avery and Heidelberger (8) on the specifically precipitable substances derived from the pneumococcus, the active substances in the latter case being carbohydrates.

Laidlaw and Dudley (9), working with the tubercle bacillus, have separated a carbohydrate material reacting with a complement-fixing serum in dilution of 1/6,000,000 from residue remaining after prolonged contact of the bacilli with alcohol and chloroform. This substance is said to be insoluble in alcohol, but whether insoluble in hot alcohol is not stated. In many respects the substance is analogous to those obtained from the pneumococcus by Avery and Heidelberger. The possibility remains open that this substance may be found associated with either the proteins or the lipoidal fractions of the tubercle bacillus, according to the circumstances of preparation.

This possibility is emphasized by the observations of Dienes and Schoenheit (10), who have returned to the examination of the lipoidal fraction and have obtained an alcohol-soluble substance, which after a preliminary purification, reacted in the complement fixation reaction in a dilution of 1/2,000,000. The alcoholic extract in the less advanced stages of purification contained carbohydrate to the extent of 26 per cent in some instances. The carbohydrate content of the more highly purified material was not determined.

The determination of the identity or otherwise of the carbohydrates of Laidlaw and Dudley with the alcohol-soluble fraction giving carbohydrate reactions as observed by Dienes and Schoenheit, is likely also to settle finally the much debated question of whether the antigenic relationships of the tubercle bacillus to the sera of animals either naturally diseased or injected one or more times with entire tubercle bacilli, are single or multiple. Till now, it may be repeated, there is no satisfactory evidence that these more natural sera react in complement fixation with any residual material after complete extraction with hot alcohol. It is conceivable, however, that such severe extraction may injure the antigenic qualities of the residue. It is also more than probable that potentially antigenic substances may remain in completely extracted residues and will be made evident by appropriate immunological procedure even though they do not react with sera prepared with the whole bacilli.

I am now able to record a simple method for the extraction, concentration and preliminary purification of the fraction soluble in hot ethyl alcohol. The procedure we have followed should serve as a most convenient starting point for further chemical manipulation of

this portion of the lipoidal fraction. I have also found that normal serum under certain conditions gives a precipitation reaction with the material. This reaction is interesting in itself and its recognition as a possible source of confusion with true immunity reactions may be important.

<center>EXPERIMENTAL.</center>

The work here recorded was all done with a rapidly growing bovine type culture of low virulence (Bovine III). Dried bacilli are extracted repeatedly in a Soxhlet extractor with absolute ethyl alcohol. When the extracts are cooled, a white precipitate forms, which is largely, although not completely, redissolved when the alcohol is again boiled. The extracts from a number of operations may be combined, boiled, filtered through paper while hot, then chilled and filtered in the ice box, giving a considerable mass of whitish paste which when quite dry can easily be ground to a powder. One or two reprecipitations under these conditions result in a powder which is completely soluble in hot alcohol to a practically colorless solution.[1] The experiments to be reported were carried out with material at this stage of purification. Weighed quantities were dissolved in hot alcohol and dilutions made from such a stock solution.

If a 0.5 to 1.0 per cent solution of this material be brought to a boil and measured with a warm pipette into 0.85 per cent NaCl solution at the temperature of the room, the 1/10 dilution is opalescent, with a bluish tinge. These solutions, or suspensions, allowed to stand for days at room or refrigerator temperature usually show no precipitation.

Such dilutions in normal saline solution freshly prepared, have been the starting point for the use of the substance in the fixation and precipitation reactions herein presented. When higher dilutions are desired, these are made by using a less concentrated alcoholic solution as a starting point, or a lesser amount of the alcoholic solution. Dilutions in series of the saline suspensions have not been practiced.

The method employed for the fixation test was fully described in our second publication. Essentially, the primary incubation is 2 hours at 37–38°, in the water bath; from 2 to 2½ units of guinea pig complement are used; approximately 10 units of hemolytic amboceptor are employed; the second incubation is ½ hour; and finally, an excess of immune serum, amounting to from 2 to 10 times the fixation unit, has been used.

When, as in this case, the antigenic value of a preparation is under consideration, the experiment may be variously interpreted. The use

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[1] I am indebted to Dr. P. A. Levene for his active interest in guiding me to this simple procedure for preliminary purification of the material.

of a definite excess of complement and a large excess of hemolytic amboceptor as outlined above made our conditions very rigid. In the same sense we have considered the end-point to be at the limit of complete fixation. Dilutions of antigen giving partial fixation have been left out of account. Under these conditions the antigenic value of an ordinary sample of the material prepared as above described tested against an immune goat serum was in the neighborhood of 1/1,300,000 dilution calculated on the weight of the powder. Reading partial fixations or otherwise relaxing the conditions of the test would make it possible to recognize the presence of the antigenic substance in two- or possibly fourfold greater dilution than this, but probably a certain degree of accuracy would be lost to comparative experiments.

As an antigen this substance has advantages in the conduct of the fixation reaction. It seems to be very stable when suspended in alcohol and if these suspensions are protected from evaporation, the preparation can be regarded as a standard one, at least for a number of months. Repeated momentary re-solution by boiling, with care to limit evaporation, does not cause deterioration to an appreciable extent. The fixation unit is usually approximately 1/100 of the anti-complementary amount. 2 fixation units give a maximum effect.

Allowing for difference in technique, it would appear that this extract in hot alcohol once or twice reprecipitated by chilling out, approaches in activity the best preparations of Dienes and Schoenheit. It is also of the order of activity of the carbohydrate of Laidlaw and Dudley. This activity together with the previously determined fact that when this substance is completely removed the bacillary bodies no longer react in the complement fixation test, seem to warrant further attention to it.

In connection with fixation studies, Calmette (11) and Caulfeild (12) have repeatedly made reference to another phenomenon spoken of as the inhibition of fixation. For the demonstration as carried out by Caulfeild quantities of the specific antigen in themselves sufficient to be anticomplementary are mixed with the immune serum and incubated with the complement. If the serum is active in the inhibitory sense hemolysis will take place when the hemolytic system is completed. This is paradoxical in that either the antigen alone, or

the antigen + serum would be expected to fix or inactivate the complement.

With the antigen preparation under discussion and sera available during the past year, I made an extra effort to demonstrate the phenomenon of inhibition. The inhibition reaction has sometimes been in evidence but the experiments were marked by many irregularities and I reached no final conclusion as to their significance. The nature of the irregularities were such as to suggest that smaller serum quantities might possibly permit the demonstration where larger quantities would fail. The time factor might also be of decisive importance. I was thus led to set up unusually long dilution series and accidentally to the interesting observation that normal serum gives a precipitation reaction with the antigen under rather unusual conditions.

The experiment fully developed after many tentative trials is as follows:

The stock alcoholic solution ($\frac{1}{2}$ per cent) of the antigen as above described, is brought to a boil and an appropriate quantity is added to 20 volumes of 0.85 per cent NaCl solution. Of this 0.9 cc. portions are put in a series of agglutination tubes. A series of serum dilutions is prepared such that when 0.1 cc. or less of each is added to the antigen in the tubes, the serum amounts will range from 0.01 cc. to 0.000002 cc. in a total volume of approximately 1 cc. The tubes are shaken and put in the ice box. The result is the formation of an abundant flocculent precipitate in a few tubes. Tubes adjacent in either direction to those showing precipitation, show an increased turbidity without the formation of flocculi. An essential condition is that the serum used shall have previously been heated to 56°C. for $\frac{1}{2}$ hour or longer. "Fresh" beef serum gives an increased turbidity without flocculation over a much wider zone. The combined results of a number of experiments with beef serum in which the above conditions were fulfilled, although at different times, are shown in Table I.

It will be noted that the precipitation centers at about concentration 0.00007, and that the turbidity change without precipitation is irregularly extended in either direction from the precipitated tubes. Serum 1116 (9-I-f) shown in Column 7 is unheated serum showing the turbidity change only.

Of the serum samples shown in this table, two numbers, 1162 and 1163, are normal, three, 1113, 1116 (13-XI) and 1118 (28-X), were from animals treated previously with avirulent bovine type bacilli,

of a definite excess of complement and a large excess of hemolytic amboceptor as outlined above made our conditions very rigid. In the same sense we have considered the end-point to be at the limit of complete fixation. Dilutions of antigen giving partial fixation have been left out of account. Under these conditions the antigenic value of an ordinary sample of the material prepared as above described tested against an immune goat serum was in the neighborhood of 1/1,300,000 dilution calculated on the weight of the powder. Reading partial fixations or otherwise relaxing the conditions of the test would make it possible to recognize the presence of the antigenic substance in two- or possibly fourfold greater dilution than this, but probably a certain degree of accuracy would be lost to comparative experiments.

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The experiment fully developed after many tentative trials is as follows:

The stock alcoholic solution (⅓ per cent) of the antigen as above described, is brought to a boil and an appropriate quantity is added to 20 volumes of 0.85 per cent NaCl solution. Of this 0.9 cc. portions are put in a series of agglutination tubes. A series of serum dilutions is prepared such that when 0.1 cc. or less of each is added to the antigen in the tubes, the serum amounts will range from 0.01 cc. to 0.000002 cc. in a total volume of approximately 1 cc. The tubes are shaken and put in the ice box. The result is the formation of an abundant flocculent precipitate in a few tubes. Tubes adjacent in either direction to those showing precipitation, show an increased turbidity without the formation of flocculi. An essential condition is that the serum used shall have previously been heated to 56°C. for ½ hour or longer. "Fresh" beef serum gives an increased turbidity without flocculation over a much wider zone. The combined results of a number of experiments with beef serum in which the above conditions were fulfilled, although at different times, are shown in Table I.

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Of the serum samples shown in this table, two numbers, 1162 and 1163, are normal, three, 1113, 1116 (13-XI) and 1118 (28-X), were from animals treated previously with avirulent bovine type bacilli,

and showing at this time moderate values in the complement fixation test. The precipitation values all approximate those of the normal animals.

TABLE I.

Serum sample*†	1163 (28-I)	1162 (28-I)	1113 (9-I)	1118 (28-X)	1116 (13-XI)	1116 (9-I-f)	1118 (26-I)
Serum amount							
cc.							
0.01	0‡	0	0	0	0	0	0
0.008	0	0	0	0	0	0	0
0.006	0	0	0	0	0	T	0
0.004	0	0	0	0	0	T	0
0.002	0	0	0	0	0	T	0
0.001	0	0	0	0	0	T	0
0.0008	0	0	0	0	0	T	0
0.0006	0	0	T	T	0	T	0
0.0004	T	0	T	T	0	T	T
0.0002	T	+++	++++	++++	T	T	T
0.0001	++++	++++	++++	++++	++++	T	++++
0.00008	++++	++++	++++	++++	++++	T	++++
0.00006	++++	++++	++++	++++	++++	T	++++
0.00004	++++	++++	++++	++++	+++	T	++++
0.00002	++++	++++	T	T	T	T	++++
0.00001	0	0	T	T	T	0	0
0.000008	0	0	T	T	0	0	0
0.000006	0	0	0	T	0	0	0
0.000004	0	0	0	0	0	0	0
0.000002	0	0	0	0	0	0	0
0.000001	0	0	0	0	0	0	0

Alcoholic extract of tubercle bacilli L. 1. 0.5 per cent hot alcoholic solution diluted 1/19 with normal salt solution. Of this 0.9 cc. used in test. Serum dilutions added to total volume 1 cc. Readings after 24 hours in ice box.

* 1162 and 1163, normal calves. 1113, 1118 (28-X), 1116, calves treated with living avirulent tubercle bacilli. 1116 (9-I-f) serum not heated. 1118 (2601) calf 4 weeks after infection with virulent culture of tubercle bacillus; died 2 weeks later.

† Except 1116 (9-I-f) all sera heated to 57–58°C. for ½ hour.

‡ ++++ = complete precipitation. T = increase in turbidity without precipitation. 0 = no change in fluid.

The reaction may be developed at either 37° or at 56°C., but it is not materially hastened or more delicate at these temperatures. If the completed reaction series is placed at 56°, the flocculi contract and

the precipitate becomes heavy almost at once, but precipitation does not occur in tubes where it was not previously present. Sometimes the reaction is not fully developed in less than 48 hours.

Following Calmette's method for the separation of his inhibitory substance from the fixing substances in immune serum, serum diluted

TABLE II.

Serum	1122 (26-I)	1122 (26-I)	1122 (26-I)	1122 (9-I)	1121 (26-I)	1121 (9-I)
Preparation	Whole serum	Supernatant fluid	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*
Serum amount *cc.*						
0.01	0†	0	T	T	T	T
0.008	0	0	T	T	T	T
0.006	0	0	T	T	T	T
0.004	0	0	T	T	T	T
0.002	0	0	++++	++++	++++	+++
0.001	0	0	++++	++++	++++	++++
0.0008	0	0	++++	++++	++++	++++
0.0006	T	0	++++	++++	++++	++++
0.0004	T	0	++++	++++	++++	++++
0.0002	T	T	++++	++++	++++	++++
0.0001	++++	++++	T	T	++++	++++
0.00008	++++	++++	0	T	++++	T
0.00006	++++	++++	0	0	+++	T
0.00004	++++	++++	0	0	T	T
0.00002	+++	+++	0	0	T	0
0.00001	T	T	0	0	0	0
0.000003	0	0	0	0	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Serum heated to 56–57° for $\frac{1}{2}$ hour before precipitation with CO_2.
† Terms as in Table I.

to 1/20 with distilled water has been precipitated with CO_2. If the CO_2 precipitate is redissolved in salt solution, and the salt content of the supernatant fluid restored, comparison shows that the CO_2 precipitate gives a similar reaction but the zone of precipitation is moved toward the stronger concentrations centering about 0.0006 instead of the tenth dilution of this amount. The fluid remaining after the

removal of the CO_2 precipitate may show no reaction whatever. In order to achieve this complete separation it is necessary that the serum be heated to 58°C. for 2 hours before the CO_2 precipitation. If CO_2 precipitation is done on fresh serum or on serum heated for a shorter time, or to 56°C., the separation is at least imperfect.

TABLE III.

Serum	1162	1162	1116	1116
Preparation	Supernatant fluid	CO₂ precipitate*	Supernatant fluid	CO₂ precipitate*
Serum amount *cc.*				
0.01	0†	T	0	T
0.008	0	T	0	T
0.006	0	T	0	T
0.004	0	T	0	T
0.002	0	++++	0	++++
0.001	0	++++	0	++++
0.0008	0	++++	0	++++
0.0006	0	++++	0	++++
0.0004	0	++++	0	++++
0.0002	0	++++	0	++++
0.0001	0	++++	d.T	T
0.00008	0	T	d.T	T
0.00006	0	T	d.T	T
0.00004	0	T	d.T	0
0.00002	d.T	0	d.T	0
0.00001	0	0	d.T	0
0.000008	0	0	0	0
0.000006	0	0	0	0
0.000004	0	0	0	0
0.000002	0	0	0	0
0.000001	0	0	0	0

* Serum heated to 58–60° for 2 hours before CO_2 precipitation.
† Terms as in Table I. d.T = questionable increase in turbidity.

Table II gives the results of serum of Calf 1122, bleeding of January 26, 1925, and compares the whole serum, the supernatant fluid after CO_2 precipitation and the redissolved CO_2 precipitate for this sample. The CO_2 precipitates of one other sample from Calf 1122 and two samples from Calf 1121 are also shown. In this experiment the whole serum, as used in the test and also as preliminary to the CO_2 pre-

cipitation, was heated to 56–57° for $\frac{1}{2}$ hour. It will be noted that the precipitations with the CO_2 precipitates are much alike. Those from Calf 1121 show a somewhat wider range. It is especially interesting that in the case of Serum 1122, the whole serum and the supernatant fluid react alike and yet the precipitate is likewise fully active.

Table III gives the results with the two samples of serum which were heated to 58–60° for 2 hours preliminary to the CO_2 precipitation. In this case the supernatant fluid is without activity while the precipitates are comparable to those shown in Table II.

The animals whose serum was used in the experiments of Table II were both infected with a virulent culture about 1 month before the samples were drawn. They had fever at this time. They had not been immunized.

The samples used in the experiments of Table III were of animals also used in those of Table I, 1162 being normal, 1116 immunized and infected.

The complement fixation reaction must, of course, be carried out with much smaller quantities of the antigen than those which show the precipitation reaction, these being anticomplementary. The fixation value of the sera in question has ranged from a maximum of 0.004 in the case of the immune sera to an immeasurable minimum of less than 0.01 in the case of the normal samples. In certain cases we have added the hemolytic system to the series after precipitation was complete, incubating the complement with the mixtures for varying lengths of time. The results have been so irregular as to be incapable of interpretation.

In one experiment, the tubes showing precipitation were pooled and filtered through paper, the filtrate being tested by complement fixation for the presence of antigen. The test was negative, showing that the specific antigenic substance with which we are dealing is included in the precipitate. The amount of serum involved in the tubes showing precipitation is much less than that required to demonstrate the fixation antibody in any of the serum samples in question and the relation of the precipitate to the immune principles of an active serum remains undetermined.

Finally the sera of a number of other species were tested; horse, sheep, goat, rabbit, guinea pig and fowl were included and all gave

precipitation. The sera were heated to 56°C. for $\frac{1}{2}$ hour before the test. The reaction zones as expressed in serum concentration vary in extent and location but no distinctive character was developed by any. These results are summarized in Table IV.

TABLE IV.

Serum* of	Horse	Sheep	Goat	Rabbit	Guinea pig	Fowl
Serum amount cc.						
0.01	0	0	0	0	0	T
0.008	0	0	0	0	0	T
0.006	0	0	0	0	0	T
0.004	0	0	0	0	0	T
0.002	0	0	T	0	T	++++
0.001	T	T	++++	0	T	++++
0.0008	T	++++	++++	0	T	++++
0.0006	++++	++++	++++	0	T	++++
0.0004	++++	++++	++++	T	++++	++++
0.0002	++++	++++	++++	++++	++++	++++
0.0001	++++	++++	++++	++++	++++	T
0.00008	++++	++++	++++	++++	++++	T
0.00006	T	T	++++	++++	++++	T
0.00004	T	++++	++++	++++	T	T
0.00002	T	T	T	T	T	0
0.00001	T	T	0	T	0	0
0.000008	T	0	0	T	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Sera heated 56–57° for $\frac{1}{2}$ hour.

DISCUSSION.

The experiments present a precipitation reaction between normal serum and an extractive preparation of the tubercle bacillus. The reaction is evidently a very delicate one considering the quantities of material involved, comparing in this respect with rather highly developed immune reactions. The quantities being adjusted on the other hand the phenomenon has the greatest constancy.

The reaction considered as an analog of immune precipitin reactions, which it certainly resembles in its main features, is of

interest in that it presents so wide a pro-zone. The serum dilutions in which activity is evident are quite beyond the range of those it would usually be thought necessary to examine in cases where the more concentrated numbers of the series failed to react, and the discovery of the precipitation was quite accidental.

The results with the fractions of serum after CO_2 precipitation, suggest some direct connection of the serum globulins with the precipitation. Conventional reasoning in consideration of the observation that in the case of serum heated to 56°C. the precipitate and the residual fluid are both active while after heating to 58°C. for a longer time, only the globulin fraction is active, would suggest that a multiplicity of substances might be involved.

The reaction may possibly be another aspect of a result obtained by Wadsworth and Maltaner (13), who found that if crude antigenic preparation from the tubercle bacillus were mixed with 5 volumes per cent of normal horse serum, and precipitated by CO_2, the antigen active in the complement fixation reaction was carried down on the globulin and could be extracted from the precipitate with alcohol.

SUMMARY.

The prolonged extraction of the tubercle bacillus with boiling ethyl alcohol, followed by one or more reprecipitations by chilling the hot alcoholic solution, easily yields a preparation very active as antigen in the complement fixation reaction.

This preparation gives a precipitation reaction with high dilution of the normal blood serum of a number of species.

The precipitation reaction presents as a peculiar feature a very long pro-zone and is further dependent on a preceding heat treatment of the serum for its demonstration.

Occurring as a reaction of normal serum, the reaction is apparently not influenced by immunization sufficient to develop moderate specific complement fixation reactions.

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STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

I. Antibody Response of Rabbits Immunized with Type III Pneumococcus.

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The absence of demonstrable type specific antibodies in the serum of rabbits immunized with Type III pneumococcus has been the common experience of investigators working with this organism (1–4). Yoshioka (5) obtained type specific agglutinins in the serum of immunized rabbits, but with irregularity. He attributed his positive results to the use of one strain of Type III, since, with two other strains, no specific antibody production was elicited. Immunization of sheep and horses with Type III pneumococcus has resulted in the development of type specific agglutinins in the sera of these animals (6), but the titre has been low and passive protection slight.

This experience with *Pneumococcus mucosus* is but a confirmation of the earlier observations (7) that the serum of animals immunized with certain of the encapsulated organisms fails to show agglutinins. This failure to demonstrate antibodies in serum under these conditions has been usually attributed to the inagglutinability of organisms possessing a mucoid capsule or to the absence of agglutinins in the test serum. Porges (8) found that if encapsulated Friedländer bacilli were first subjected to a process of decapsulation, they then became agglutinable in immune serum. Hanes (1) reported that Type III pneumococci were agglutinated in their homologous immune serum following decapsulation of the organisms by means of the Porges method.

In the course of a series of investigations on immunity to Type III pneumococcus, the response of rabbits to immunization with this organism was investigated.

EXPERIMENTAL.

Antigen.—Three different strains of Type III pneumococcus were employed. These strains were derived from the blood of patients suffering from lobar pneumonia and possessed all the characteristics of Type III. They were Grampositive diplococci; they showed capsule formation when suitably stained; they grew on blood agar plates with the production of large mucoid colonies; they were bile-soluble; they agglutinated promptly in Type III antipneumococcus horse serum; they were virulent for mice in 0.0000001 cc. doses. Both living organisms and vaccine were used for injection. A 16 hour plain broth culture was used for the living organisms. The vaccine was made by centrifuging such a culture, resuspending the organisms in physiological salt solution, and heating at 56° for half an hour.

Methods of Immunization.—Three methods of immunization were employed. (1) The intravenous injection of vaccine according to the method described by Cole and Moore (9). This method consists in alternating for 6 weeks a week of daily injections of 1 cc. of vaccine with a week of rest. (2) Following a week of intravenous vaccine injections of 1 cc. each day, living organisms were injected at 3 to 4 day intervals in increasing amounts from 0.1 to 10 cc. over a period of 4 weeks. (3) Living organisms were injected intravenously from the beginning of immunization; 1 cc. was given daily for 6 days followed by increasing doses from 3 to 10 cc. at 3 to 4 day intervals over a period of 4 weeks.

All animals were bled 10 to 12 days after the last immunizing dose.

Agglutinin Reaction.—0.5 cc. of a saline suspension of heat-killed organisms was added to an equal volume of the serum dilutions. In the tests with type specific pneumococci, a positive reaction, if present, usually appeared immediately and the final reading was made after the tubes had remained 2 hours in the water bath at 37°C. When decapsulated Type III pneumococci or R strains of pneumococci were used, the reading was made after the tubes had remained 2 hours in the water bath at 37°C. and overnight in the ice box.

Precipitin Reaction. Nucleoprotein.—The nucleoprotein solutions of Pneumococcus used as precipitinogen in the tests were made by the method described by Avery and Morgan (10). They contained approximately 3 mg. of N per cc. 0.2 cc. of the test serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the nucleoprotein solution. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

Soluble Specific Substance.—This substance, representing the N-free carbohydrate derived in a purified state from Type III pneumococci according to the method described by Heidelberger and Avery (11), was used in dilutions of 1–10,000, 1–20,000, 1–40,000, and 1–100,000. Dilutions of this degree were employed in order to escape the inhibition zone experienced (12) when higher concentrations are tested. 0.2 cc. of serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the different dilutions of the soluble specific substance. The precipitin reaction is usually immediate; however, the final reading was made after 2 hours in the water bath at 37°C.

Passive Protection Tests.—These tests were carried out in mice by the simultaneous injection intraperitoneally of 0.2 cc. of serum and culture dilution, according to the method previously described (13).

The results obtained in the experiments to be reported involve the activities of two antigen-antibody systems. In order to avoid confusion as to nomenclature, certain terms used throughout the body of the report will be defined. It has been shown by Avery and Heidelberger (14), that pneumococci are capable of stimulating the production of two distinct antibodies, depending in a large measure on the nature of the bacterial substances used as antigens. An intact pneumococcal cell possessing the soluble specific substance, when employed antigenically, stimulates the production of antibodies which are reactive with the homologous soluble specific substance. If the soluble specific substance is present in the cells used in the agglutination test, type specific agglutination occurs; if the soluble specific substance is present in solution, precipitation of this substance occurs. The antibody, which is capable of agglutinating the type specific Pneumococcus and of precipitating the type specific soluble substance will be designated as *anti-S*. The evidence of all previous work indicates that the efficacy of serum in the passive protection of mice against pneumococcus infection depends on the presence in the serum of anti-S.

The second antigen-antibody system involves the nucleoprotein fraction of the cell and the antibody response elicited by it. Pneumococcus nucleoprotein, in contrast to the soluble specific substances, is without type specificity. The degraded R strains of pneumococci also are without type specificity. Therefore the use, antigenically, of either nucleoprotein substance or R strains results in the production of non-type specific antibodies, which precipitate the nucleoprotein derived from any Pneumococcus and agglutinate all R strains. These antibodies will be designated as *anti-P*.

I. Agglutinins.

Absence of Agglutinins for the Encapsulated Type III Pneumococcus in the Sera of Animals Immunized with Type III Pneumococcus.

Twenty-eight rabbits were used in these immunization experiments. Twenty received vaccine alone; six received vaccine and living organ-

isms; two received living organisms alone. Their sera were then tested for the presence of agglutinins against the encapsulated Type III pneumococcus. Out of the twenty-eight rabbits, the sera of twenty-four failed to show type specific antibodies (anti-S). In the four positive sera type specific antibodies (anti-S) were present in low titre and in only one was the agglutinin reaction positive in a dilution of 1 to 20 (Table I). Such results are confirmatory of the experience of others in working with Type III and are in striking contrast to the results obtained when rabbits are immunized with Pneumococcus Type I or Type II, whereby type specific antibodies are readily demonstrable in the immune sera.

Presence of Agglutinins for R Strains of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

In demonstrating the twofold antigenicity of pneumococcus substances, Avery and Heidelberger (14) showed that when intact pneumococcal cells are used as antigen the resultant antibodies consist chiefly of type specific agglutinins (anti-S) for the homologous organism; when ruptured cells or solutions of pneumococci are used as antigen the antibody response is predominantly of the anti-P character. Since the sera of rabbits immunized with Type III pneumococcus fail, in most instances, to show anti-S antibodies, and in view of the dual antigenic nature of pneumococci, these immune sera were tested for the presence of anti-P antibodies. Anti-P antibodies are reactive against the degraded R strains of Pneumococcus. As has been brought out by Reimann (15, 16) R strains are avirulent, non-encapsulated, and non-type specific. They may be derived from any of the fixed types of Pneumococcus and are agglutinated by any anti-P serum. Therefore, R strains of pneumococci are adequate in testing for the presence of anti-P antibodies. The results recorded in Table I were obtained by testing the sera of rabbits, immunized with Type III pneumococcus, for the presence of anti-P antibodies, as evidenced by the agglutination of an R strain. The sera of twenty-seven of twenty-eight rabbits possessed anti-P antibodies.

The results of this experiment indicate that rabbits immunized with Type III pneumococcus—even though this organism is one of the fixed types—react in the majority of instances by the production, not

of the type specific anti-S antibody, but of the anti-P antibody. The immunological response is identical with that obtained following immunization with R strains or solutions of pneumococci. The inference is, then, that Type III pneumococci are so altered, following introduction into the animal body, that the type specific antigen is made ineffectual and the nucleoprotein is liberated to stimulate the production of anti-P antibodies.

Agglutination of Decapsulated Pneumococci in the Sera of Rabbits Immunized with: (1) Type III Pneumococcus, (2) R Strains of Pneumococcus, (3) Nucleoprotein Derived from Pneumococcus.

Hanes (1), as previously stated, showed that Type III pneumococci, deprived of their capsule by means of the Porges (8) method, were agglutinated in the sera of rabbits immunized with Type III pneumococci; whereas, when the encapsulated organisms were tested in the same immune sera, no agglutination occurred. Both Porges and Hanes infer that the inagglutinability of encapsulated organisms is due, not to the absence of agglutinins in the test serum, but to the presence of the capsule around the organism. As proof of this they show that removal of the capsule is followed by agglutination.

Since it has been shown in Table I that rabbits immunized with Type III pneumococcus possess anti-P antibodies and, since the chief characteristic of anti-P antibodies is the agglutination of non-encapsulated pneumococci, an explanation of the results of Hanes is made possible. R strains of pneumococci agglutinated by Type III immune rabbit serum (Table I) are organisms which have been deprived of their capsule by cultural methods. The Type III organisms which Hanes found agglutinable were deprived of their capsule by a chemical procedure. The agglutination of both of these non-encapsulated organisms is identical (Table I) in Type III immune rabbit serum. Their immunological identity is further brought out by testing the agglutination of chemically decapsulated organisms in the sera of rabbits immunized with R strains and with pneumococcus nucleoprotein. The results (Table II) show that any anti-P serum is capable of agglutinating the chemically decapsulated Type III pneumococcus and R strains equally well. In reports on Friedländer bacilli Julianelle (17) obtained similar results; namely, that decapsulated and R

TABLE I.

Agglutination of Encapsulated Type III Pneumococcus, Decapsulated Type III Pneumococcus, and of an R Strain of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Agglutinins for Pneumococcus III encapsulated — Serum dilutions					Agglutinins for Pneumococcus III decapsulated — Serum dilutions					Agglutinins for R₃ strain of pneumococcus — Serum dilutions					
	1-2	1-10	1-20	1-40	1-80	1-10	1-20	1-40	1-80	1-160	1-2	1-10	1-20	1-40	1-80	1-160

23	—	—	—	—	—					++++	++++	++	+	±	—
24	—	—	—	—	—					++++	++++	+++	++	+	±
25	—	—	—	—	—					++++	+++	++	+	—	±
26	—	—	—	—	—					++++	++++	++	+	±	—
27	××××	××	X̲	—	—					++++	+++	+	±	—	—
28										++++	++++	++	+	±	—

Controls: 0.5 cc. organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. organisms + 0.5 cc. of normal rabbit serum: negative.

×××× indicates characteristic compact disc of type specific agglutination with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates disc which can be partially broken up with cloudy supernatant fluid. × indicates coarse granulation. X̲ indicates doubtful granulation. — indicates negative.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. — indicates negative.

TABLE II.

Agglutination of Decapsulated Type III Pneumococci and of R Pneumococci in the Sera of Rabbits Immunized with Two Different R Strains of Pneumococcus and with Pneumococcus Nucleoprotein.

Rabbit No.	Immunized with	Decapsulated Type III						R₃ pneumococci					
		1-10	1-20	1-40	1-80	1-160	1-320	1-10	1-20	1-40	1-80	1-160	1-320
29	R_1 strain	++++	+++++	+++	+	±	−	++++	+++	++	+	±	−
30	R_2 strain	++++	+++	+	±	−	−	++++	+++	+	−	−	−
31	Nucleo-protein	++++	+++	++	+	±	−	++++	+++	++	+	−	−

Controls: 0.5 cc. of organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. of organisms + 0.5 cc. of normal rabbit serum: some sedimentation which was made diffuse by slight tapping.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

strains of Friedländer bacilli were identical in their agglutination reactivity in anti-P (Friedländer) serum.

II. Precipitins.

It was shown by Reimann (16) that sera which contained agglutinins (anti-P) for R strains of Pneumococcus also contained precipitins for pneumococcus nucleoprotein solutions. Therefore, since the sera of the rabbits immunized with Type III pneumococcus possessed anti-P agglutinins, they were tested for the presence of precipitins for pneumococcus nucleoprotein (Table III).

A comparison of Table III with Table I will show that all the sera which contained anti-P agglutinins also possessed anti-P precipitins. Since the nucleoprotein in this test was obtained from a Group IV pneumococcus, the possibility of a type specific reaction is excluded and the experiment further identifies the antibody present in the Type III immune sera as of an antiprotein character.

The sera of the rabbits immunized to Type III were also tested for the presence of precipitins against Type III specific soluble substance (Table III). As has been shown by Avery and Heidelberger (14), the type specificity of pneumococci depends upon the presence of this substance. They have obtained it in a highly purified state from each of the three fixed types of Pneumococcus. Immune sera which possess type specific agglutinins (anti-S) for pneumococci of a fixed type also possess precipitins for the soluble specific substance derived from that type. The results obtained with Type III immune rabbit sera used in these experiments show the concomitant occurrence of Type III specific agglutinins (anti-S) and precipitins for the Type III soluble specific substance. On the other hand, those sera which failed to show type specific agglutinins (anti-S) also failed to precipitate the soluble specific substance. This is further evidence that the lack of agglutination of the encapsulated Type III cell in homologous immune rabbit serum is due to the actual absence of the anti-S antibody in demonstrable quantity since such factors as were supposed to render the encapsulated organism inagglutinable are not present in the precipitin test.

TABLE III.

Precipitation of the Soluble Specific Substance of Type III Pneumococcus and of a Solution of Pneumococcus Nucleoprotein in the Sera of Rabbits Immunized with Type III Pneumococcus.

	Precipitins for soluble specific substance of Type III pneumococcus				Precipitins for solution of pneumococcus nucleoprotein
Rabbit No.	Dilutions of soluble specific substance				Dilution of protein
	1–10,000	1–20,000	1–40,000	1–100,000	1–300
1	−	−	−	−	+++
2	−	−	−	∼	++
3	−	−	−	−	++
4	−	−	−	−	++
5	−	−	−	−	+++
6	−	−	−	−	++
7	−	−	−	−	++
8	−	−	−	−	+++
9	−	−	−	−	++
10	−	−	−	−	++
11	−	−	−	−	++
12	−	−	−	−	++
13	−	−	−	−	++
14	−	−	−	−	++
15	××××	××	×	×	+
16	××××	×	×	−	±
17	×××	×	−	−	++
18	−	−	−	−	++
19	−	−	−	−	++
20	−	−	−	−	−
21	−	−	−	−	+
22	−	−	−	−	++
23	−	−	−	−	++
24	−	−	−	−	+++
25	−	−	−	−	++
26	−	−	−	−	++
27	××××	×	×	−	++
28	−	−	−	−	+++

Controls: 0.5 cc. of various dilutions of soluble specific substance + 0.5 cc. salt solution: clear. 0.5 cc. of various dilutions of soluble specific substance +0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: clear. 0.5 cc. of pneumococcus nucleoprotein solution + 0.5 cc. of salt solution: slightly hazy

III. Passive Protection in Mice.

The sera of the twenty-four rabbits immunized with Type III pneumococcus were tested for their ability to protect mice against infection with Type III pneumococcus. The dependence of passive protection on the presence of type specific antibodies (anti-S) in the test serum is well known to investigators working in experimental pneumococcus immunity. Therefore, it is to be expected that those sera containing anti-S afford some protection and that those without it fail. The results recorded in Table IV show that protection parallels the presence of anti-S and not anti-P. Of the twenty-four sera tested, the four possessing anti-S showed protection; of the twenty possessing no demonstrable anti-S, seventeen afforded no protection, and three afforded very slight protection. The explanation of the results obtained with the latter three sera probably rests on the fact that these sera contained sufficient type specific antibodies (anti-S) to afford some protection but not sufficient to be demonstrable by test-tube agglutination. That the mouse protection test is a more delicate test for the presence of anti-S than test-tube agglutination may be brought out by the use of Type I antipneumococcus serum. This serum may be diluted to a point where no test-tube agglutination is demonstrable and yet such a dilution of serum affords some protection. A further report will be made later on the results obtained in passive protection following the use of the sera of rabbits immunized with Type III pneumococci.

An analysis of the antibodies present in the sera of twenty-eight

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0.5 cc. of pneumococcus nucleoprotein solution + 0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: slightly hazy.

× × × × indicates characteristic compact disc of type specific precipitin reaction with clear supernatant fluid. × × × indicates compact disc with faintly cloudy supernatant fluid. × × indicates smaller disc at bottom of tube with cloudy supernatant fluid. × indicates cloudy fluid. × indicates very faintly cloudy fluid. − indicates negative.

+ + + indicates precipitate at bottom of tube with faintly cloudy supernatant fluid. + + indicates slight precipitate with cloudy supernatant fluid. + indicates cloudy fluid. ± indicates very faintly cloudy fluid. − indicates negative.

rabbits immunized with Type III pneumococcus may be summarized as follows:

Agglutinins.—Twenty-four failed to show type specific agglutinins (anti-S). Four possessed type specific agglutinins (anti-S) in low titre. Twenty-seven possessed anti-P agglutinins in appreciable titre. One failed to show evidence of any antibody response.

TABLE IV.

Summary of Passive Protection in Mice by the Use of Sera of Rabbits Immunized with Type III Pneumococcus.

	Demonstrable anti-S antibodies		Demonstrable anti-P antibodies		Rabbit No.	Passive protection in mice against Type III pneumococcus infection.			
						Dose of culture			
	Agglutinins	Precipitins	Agglutinins	Precipitins		0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Sera of 17 rabbits	Absent	Absent	Present	Present		D.	D.	D.	D.
Sera of 3 rabbits	Absent	Absent	Present	Present	6	D.	D.	D.	S.
					11	D.	D.	D.	S.
					12	D.	D.	S.	S.
Sera of 4 rabbits	Present	Present	Present	Present	15	S.	S.	S.	S.
					16	S.	S.	S.	S.
					17	D.	S.	S.	S.
					27	D.	S.	S.	S.

D. indicates death of animal.
S. indicates survival of animal.

Precipitins.—The presence of precipitins for Type III soluble specific substance paralleled the presence of type specific agglutinins. The presence of precipitins for pneumococcus nucleoprotein paralleled the presence of anti-P agglutinins.

Passive Protection in Mice.—Four sera possessing demonstrable anti-S antibodies afforded some protection against Type III infection.

Of twenty sera without demonstrable anti-S, seventeen failed to confer any protection; three afforded minimal protection.

DISCUSSION.

The common experience that Type III pneumococci, antigenically employed, fail to stimulate the production of type specific agglutinins (anti-S) has been encountered in a great majority (85.8 per cent) of the rabbits used in the experiments here reported. It is a striking fact that such a cell, possessing a large amount of soluble specific substance, and highly virulent for mice, acts so feebly in stimulating type specific antibodies (anti-S); whereas Type I and Type II pneumococci, possessing the same qualities, are so effective in producing homologous type specific antibodies. However, the presence of agglutinins for R pneumococci and precipitins for pneumococcus nucleoprotein (anti-P antibodies) is evidence that Type III pneumococci are not without an antigenic component. The results of these experiments reveal the fact that the antibody response of rabbits to immunization with the encapsulated Type III pneumococcus is identical with the antibody response obtained by immunization with solutions of pneumococci. In both instances the production of anti-P antibodies has been stimulated. Such a result could be accomplished only by *in vivo* disruption of the pneumococcal cells. The inference, then, may be drawn that normal rabbits possess a mechanism whereby Type III pneumococci, following intravenous injection, are disintegrated in such a manner that the part of Type III antigenic complex, which stimulates type specific agglutinins (anti-S), is destroyed. The nucleoprotein fraction of the cell, however, remains capable of stimulating anti-P antibodies.

The identification of the antibody present in Type III immune rabbit serum, as being anti-P, is explanatory of Hanes' results with decapsulated Type III pneumococci. By decapsulation, Type III pneumococci are reduced to an R form, and are agglutinated by anti-P antibodies. The failure of encapsulated Type III to agglutinate is due to the actual absence of anti-S antibodies.

CONCLUSIONS.

1. Type III pneumococci fail in the majority of instances to stimulate the production of anti-S antibodies. (Type specific agglutinins, type specific precipitins, and antibodies affording type specific protection in mice.)

2. Type III pneumococci are effective in the stimulation of the production of anti-P antibodies (agglutinins for R strains of pneumococci and precipitins for pneumococcus nucleoprotein). These antibodies are ineffectual in the passive protection of mice.

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INFLUENCE OF LIGHT ON THE GROWTH AND MALIG NANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

Second Paper.

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The experiments reported in the present paper are a continuation of previous work dealing with the effect of light on the growth and malignancy of a transplantable epithelial neoplasm of the rabbit (1) and are a part of a general study on the relationship of external conditions to the physical constitution of normal rabbits and to the reaction of rabbits to certain experimental diseases.

The belief that there is a relationship between sunlight and the manifestations of this particular tumor arose, first, from the fact that a characteristic feature of the disease is the seasonal variability of its behavior and, second, that a correlation could be drawn between the prevailing level of sunshine and changes in the curve of sunlight, on the one hand, and variations in malignancy of the tumor process, on the other (2). In order to test this belief, experiments were carried out in which conditions of light could be controlled. The results of the first experiment (1) showed that, as compared with rabbits living under ordinary indoor conditions of diffused sunlight, a slightly lower plane of malignancy obtained in a group of rabbits kept in constant darkness but that a very low level prevailed in the group exposed to constant light. The particular type of lighting (Mazda lamps and mercury arcs in crown glass) employed in this experiment, however, precluded its use except during the colder months, so that a different arrangement was necessary to permit experiments at other seasons of the year.

In the present paper, 4 experiments are reported in which were

studied the effect of constant illumination with Cooper Hewitt mercury arcs in crown glass and the effect of constant darkness upon the general course and manifestations of the malignant disease.

Methods and Material.

The experiments were carried out from October 1, 1925, to April 12, 1926. 3 animal rooms with similar environmental conditions other than those of lighting were employed. These rooms which will be referred to as the light, the dark and the control or unaltered room, respectively, have already been described in connection with the 1st experiment (1). It is sufficient in the present report merely to call attention to the lighting arrangement of the light room which was the only feature in the equipment of the 3 rooms that was changed.

A *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. All other light was excluded. The rabbit cages were placed in racks on either side of and parallel with the mercury arcs. The intensity of light reaching the cages was not entirely uniform, those in the central portions of the racks receiving more light than those at the top and bottom or at the ends. The average intensity was 200 foot candles.

The spectrogram of the mercury arcs in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside air.

The rabbits employed were males, chosen from a selected stock and were matched as to breed and type. The majority were approximately 8 to 12 months old. Each animal was separately caged and all were fed a similar diet of hay, oats and cabbage.

The experiments were all conducted in the same manner. Comparable groups of rabbits were placed in each room at the same time where they remained for the duration of the experiment. After varying intervals, each group was inoculated with the tumor. All inoculations were made in one testicle with 0.3 cc. of an emulsion of an actively growing primary tumor. This neoplasm is considered to be of epithelial origin (3).

Frequent examinations of the rabbits were made, special attention being given to the general physical condition of the animals, the character of the primary tumor and the detection of metastases recognizable during life. In addition, the rabbits were weighed at regular intervals. Certain animals which developed signs and symptoms of impending death were killed by an injection of air into the marginal ear vein. The experiments were terminated 2 months after inoculation at which time all surviving animals were killed. A complete postmortem examination of each rabbit was made with special reference to the condition of the primary tumor,

the incidence of metastatic tumors and the number, distribution, size and state of these growths.

The observations thus obtained have been used as a basis for evaluating the effect of the particular light condition upon the general level of malignancy. The results are considered from a group standpoint in order to minimize the factor of individual animal variation.

The method used in analyzing the data on metastatic foci obtained at post-mortem examination requires some explanation. First, the sites or foci of secondary tumors, not their actual number, are counted and hence the expressions "foci of metastases," "metastatic rate" or "distribution of metastases." On the other hand, the number of metastases detected during life refers to the actual number found.

Second, in order to classify the character of the disease in such general terms as high, moderate and low levels of malignancy, the metastatic foci have been grouped in the following divisions:

I. Suprarenals and eyes................................ 4 possible foci
II. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum.. 19 " "
III. Lungs and pleura, liver, kidneys and pancreas........... 5 " "
IV. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system.... 30 " "

The reasons for this classification have been stated in the paper describing the results of the first experiment (1). Suffice it to say here that in instances of low malignancy, metastases may be found only in the suprarenals and eyes, while in cases of high malignancy with death in 3 to 5 weeks after inoculation there may be a widespread distribution of tumor to many tissues and organs including the skin, muscles, bones and glands of internal secretion which are practically never involved in instances of low malignancy. An occasional exception to this rule is met with in certain cases of tumors in the cranial sinuses or facial bones which may be the only metastases found.

In the third place, the distribution of metastases has been considered upon a percentage basis of the possible sites of such growths as determined by the actual location of metastases in the first 20 generations of the tumor (4). The percentage involvement for each rabbit was first obtained and these values were then combined on a group basis to form what have been called "summation" values of metastatic foci.

Results.

The results of the 4 experiments consisting of clinical and post-mortem observations are presented in 4 tables and 2 charts, the latter being graphic representations of the distribution of metastatic foci. (Tables I, II, III and IV; Text-figs. 1 and 2.) The experiments are arranged according to the length of exposure to constant illumination or constant darkness prior to inoculation, that is, Nos. I and II, 4 weeks; No. III, 5 weeks and No. IV, 16 weeks. Since the duration of observation subsequent to inoculation was in each case 2 months, the entire period of exposure to these conditions was as follows: Nos. I and II, 12 weeks; No. III, 13 weeks and No. IV, 24 weeks. The results of each experiment are presented in the order of the control, the dark and the light groups, respectively.

Analysis and Discussion of Results.

The purpose of these experiments was to determine whether the constant exposure of rabbits to light supplied by mercury arcs in crown glass and to continuous darkness influenced the general course and character of a malignant tumor. Recognizing the fact that an adequate interpretation of results obtained in experiments of this nature implies a consideration of the tumor process in as comprehensive a way as possible, the present analysis has included all features of the disease which are believed to be significant. In addition, the analysis has been made on a group basis in order to avoid individual animal variation, and the results obtained under the experimental light conditions have been compared, in each instance, with those of appropriate control groups. It will aid to a clearer understanding of the evaluation of these comparisons if a brief description of the disease in the 4 control groups be given first.

General Character of the Disease.—The most striking feature of the tumor process in the control groups was the relatively low level of malignancy which prevailed in all 4 experiments. There was a considerable variation in disease severity among the groups but in none of them was the average plane of malignancy high.

As far as general procedure was concerned, the experiments differed in only 2 particulars, that is, in the time of year at which

they were carried out and second, in the length of exposure to constant illumination and to constant darkness before inoculation which, of course, does not concern the control animals. The dates of inoculation were as follows:

Experiment I, October 27, 1925.
" III, November 2, 1925.
" IV, January 29, 1926.
" II, February 11, 1926.

The most pronounced disease developed in Experiments I and III but the general plane of malignancy was low as compared with many series observed in previous years. An even less severe disease occurred in the 2nd and 4th experiments, and in the case of No. II, the condition was extremely mild. The generally low level of malignancy as well as the group variations in disease severity are well illustrated by the differences in the mortality rates and in the incidence of metastases determined at postmortem examination as shown by the following data:

	No. of rabbits	Mortality rate	Incidence of metastases
		per cent	*per cent*
Experiment I..........................	11	36.4	81.9
" III..........................	11	27.3	63.6
" IV..........................	5	20.0	40.0
" II..........................	10	0	30.0
Weighted average......................	37	21.6	56.8

Similar variations in metastatic incidence and in the death rate were noted in a study of the first 20 generations of the tumor (5) but there were certain peculiarities of metastatic distribution in the present experiments, which were not wholly characteristic of the disease as previously observed. While this phase of the subject cannot at present be discussed in detail, its occurrence should be noted and its most pronounced feature, namely, metastases to the bones, briefly described because of its bearing upon the experimental results obtained.

As compared with the first 20 generations of the tumor, the incidence and distribution of bone metastases were as follows:

	First 20 generations, tumor	Controls of these experiments
Total No. of rabbits	191	37
Incidence of bone metastases	15 or 7.9 per cent	6 or 16.2 per cent
Proportion of bone metastases to total foci	13.2 per cent	11.6 per cent
Distribution of bone lesions		
Facial bones	12	16
Calvaria...........................	6	0
Thorax............................	4	1
Posterior extremities	4	2
Spinal column	3	1
Anterior extremities	1	0
Total bone lesions..................	30	20

Although the disparity in the numbers of animals in the 2 series and the mixed nature of the material comprising the first 20 generations does not strictly permit a comparison, still there are 2 particulars in which the control rabbits of these experiments differed from the larger group. In the first place, there was a much higher incidence of bone lesions—16.2 as contrasted with 7.9 per cent and second, an increase in the proportion of metastases to the facial as compared with other bones (80.0 per cent as contrasted with 40.0 per cent). The ratio of bone metastases to the total number of secondary growths, however, was practically the same in both series. From the standpoint of animal incidence, the bone lesions of the large series were almost equally divided between cases of fulminating malignancy and animals with relatively malignant but slowly progressive tumors. Among the present controls, bone lesions occurred only in cases of comparatively pronounced malignancy. There were no instances in the controls of the 2nd type of malignancy, which is another point in which the character of the disease in these experiments differed from that previously observed. The tumor process of the control rabbits fell, on the whole, into 2 classes, in 1 of which malignancy was fairly well marked while in the other, the disease was very mild as evidenced by the absence of, or the few numbers of, metastases found.

The character of the disease in the groups exposed to constant illumination and to constant darkness did not entirely conform in its general picture to that of the controls, and these differences will be pointed out in the following analysis of the results obtained in these groups.

TABLE I.

Analysis of Clinical Observations.

Experiment No.	Length of exposure before inoculation	Group	No. of rabbits	Primary tumors	Metastases			Mortality		
					Incidence		Total No.	No.	Rate	Time after inoculation
					No.	Per cent				
	wks.								*per cent*	*wks.*
I	4	C.	11	All +	5	45.5	13	4	36.4	4, 5½, 6½*, 7½
		D.	12	All +	4	33.3	7	3	25.0	5, 6, 7½
		L.	12	All +	3	25.0	8	1	8.3	3
II	4	C.	10	4 +	0	——	0	0	——	
		D.	9	2 +	2	22.2	2	1	11.1	7
		L.	10	0 +	0	——	0	0	——	
III	5	C.	11	All +	3	27.3	4	3	27.3	4, 5, 5½
		D.	12	All +	2	16.7	4	2	16.6	4, 6
		L.	10	All +	4	40.0	9	3	30.0	4½, 5, 5½
IV	16	C.	5	2 +	2	40.0	10	1	20.0	6½
		D.	5	2 +	2	40.0	4	1	20.0	6½
		L.	4	2 +	0	——	0	0	——	

C. = controls; D. = groups exposed to constant darkness; L. = groups exposed to constant illumination.

* Complicating empyema.

Clinical Observations.—Under this heading are discussed the primary tumor, the development of metastases recognizable during life and the general physical condition of the animals.

A primary tumor developed in all animals of Experiments I and III (Table I). In the others, however, there were a number of instances as shown below in which no definite primary growth occurred.

	Experiment IV	Experiment II
	per cent	*per cent*
Controls	60.0	60.0
Darks	60.0	77.8
Lights	50.0	100.0

Although the intratesticular route of inoculation has yielded an almost uniform incidence of primary tumors, there have been instances in which none developed. An error in technic with the inoculum deposited in the scrotal sac may have been responsible for some of these failures, but in 2 series inoculated in the fall of 1922, the number of negative results were comparable to those of the present experiments (4). In addition, it may be noted that as far as Experiment IV was concerned, the rabbits had been in the laboratory 6 months prior to inoculation and 4 months under the conditions of the experiment—a period probably sufficient for the establishment of a relatively stable state of the organism. It would appear, and this point will later be taken up in greater detail, that the factors of stability or changeability as applied to external conditions are of prime importance in influencing tumor malignancy, the first manifestation of which is the growth capacity of the transplant.

It was also observed in many rabbits kept in the light room that the testicles were reduced in size and became firmer, conditions which would not necessarily be conducive either to initial or to continued tissue growth. Perhaps this factor in connection with the low degree of malignancy prevailing at the time was largely responsible for the completely negative results as regards the primary tumor in the light group of Experiment II.

The non-development of a primary tumor, however, was not necessarily followed by an absence of growths in other parts of the body as shown by the following data compiled from Experiment II.

	Total No of rabbits	Negative primary results	Incidence of metastases with no primary tumors
Controls	10	6	1 or 16 7 per cent
Darks	9	7	2 " 28.6 " "
Lights........................	10	10	1 " 10 0 " "

The character of growth of the primary tumor differed under the various light conditions of the experiments. On the whole, the largest tumors developed in the controls, and in general these groups showed a definite tendency towards a longer persistence of active primary growth as compared with an earlier regression in the tumors of the rabbits from the dark and light rooms. The most irregular type of growth occurred in the rabbits kept in the dark room. During the 1st week after inoculation, little difference was noted between the primary tumors of these rabbits and those of the controls, but in the 2nd and 3rd weeks, the resemblance ceased. It then became difficult to determine from clinical observation whether certain tumors in the dark series were growing. Eventually, some tumors grew more rapidly than those in the controls, but in the majority of instances the speed and degree of growth were less marked.

In the groups kept in the light room, on the other hand, the initial development of the primary tumor was definitely slower and less pronounced than in the controls. The majority of the tumors in these groups did not attain the size of those in the controls and in addition, regression took place earlier and was somewhat more rapid. There were certain instances, however, of very actively growing and extensive primary tumors, but they were not as numerous as in either the dark or the control groups.

The condition of the primary tumors at postmortem examination was, on the whole, in harmony with the general character of the disease at the time this was done, irrespective of the group to which the animal belonged. Thus, in rabbits dying within 4 to 6 weeks after inoculation and in which metastases were found to be more or less widely distributed, the primary tumors showed variable amounts of living tissue. On the other hand, the growths in the animals living to the end of the observation period were in the majority of instances either healed or largely necrotic and heavily encapsulated.

The incidence of metastases recognizable during life among control groups varied in the different experiments from 0 to 45.5 per cent (Table I). While there was only 1 control group (Experiment II) in which no secondary tumors were detected, there were 2 such groups from the light room (Experiments II and IV) and the incidence in the others was 25.0 and 40.0 per cent. There was less variation

among the groups kept in the dark room, the values being 16.7 to 40.0 per cent. If the experiments are combined, the incidence of "clinical" metastases was:

	Total No. of rabbits	No. of rabbits with metastases	Rate
			per cent
Controls.....................................	37	10	27.07
Darks.......................................	38	10	26.31
Lights.......................................	36	7	19.44

The numbers of secondary tumors detected during life were—Controls 27, Darks 17, Lights 17. There was 1 rabbit from the light room (Experiment III) in which a malignant disease developed and the numerous growths in superficial parts of the body of this rabbit considerably augment the total number of clinical metastases of the light groups. On the other hand, there was observed among these rabbits the unusual occurrence of regression of metastases in superficial parts of the body. The tumors of the iris in 2 rabbits healed during the period of observation and in a 3rd animal they were entirely necrotic at postmortem examination.

Continuous exposure to constant illumination of mercury arcs in crown glass for 3 to 6 months did not appear to induce any outspoken deleterious effect upon the general state of health. All rabbits were given a superabundance of food and those in the light room generally ate more than those in the dark or control rooms, particularly during the first weeks of the experiment. The evidence furnished by body weight determinations, however, indicates that conditions of constant darkness were less favorable than those of constant illumination or of variable diffused sunlight. The changes in mean body weights expressed in percentage values of the initial weights are shown in Table II. The first figures for each experiment include all the rabbits in each group while the second values represent only those animals which survived the observation period of 2 months. On the basis of both computations, the gains in body weight of the groups in the light room were approximately the same or exceeded those in the control room while in only 1 experiment, III, was this the case with the rabbits under conditions of darkness, and here the greater gain

of the surviving rabbits was not as much as that of the surviving animals from the light room. In the other 3 experiments, the groups from the dark room gained much less weight than the controls.

These observations are in general agreement with those obtained in previous experiments in which normal rabbits (6) and rabbits inoculated with *Treponema pallidum* (7) or with the tumor (1) were exposed to conditions of constant light or of constant darkness.

Mortality.—The mortality rates of the control groups ranged from 36.4 per cent in Experiment I to 0 per cent in Experiment II as is seen

TABLE II.

Mean Body Weight. Percentage Variations.

		Controls	Lights	Darks
		per cent	per cent	per cent
I	All rabbits	+27.9	+24.5	+11.1
	Survivors	+25.9	+24.1	+22.5
II	All rabbits	+10.6	+12.6	+ 2.2
	Survivors	+10.6	+12.6	+ 6.8
III	All rabbits	+22.4	+24.8	+23.0
	Survivors	+20.9	+34.9	+26.4
IV	All rabbits	+33.5	+39.0	+23.2
	Survivors	+35.4	+39.0	+17.3

in Table I. The groups from the dark room showed less variations in this respect, that is from 25.0 to 11.1 per cent, and, as will be recalled, this was also the case in regard to the development of metastases in superficial parts of the body. In 2 experiments, the rates were lower than those of the corresponding control groups (I and III), in another it was the same (IV), while in the last, it was slightly higher (II).

The mortality rates of the light groups also varied considerably, from 30.0 to 0 per cent, but the nature of this variation differed from that of the controls in that there were no deaths in 2 of the groups from the light room while the rates for the other groups were 8.3 and 30.0 per cent as compared with 36.4 and 27.3 per cent for the controls.

The combined mortality rates for the 4 experiments were as follows:

	No. of rabbits	No. of deaths	Mortality rate
			per cent
Controls...................................	37	8	21.6
Darks.....................................	38	7	18.4
Lights....................................	36	4	11.1

From these figures as well as from those of individual experiments, it is seen that the highest death rate occurred among the controls and the lowest in the groups kept under conditions of constant illumination while the rate for the groups from the dark room approximated that of the controls.

The great majority of the rabbits in the 4 experiments survived the observation period of 2 months, but from the distribution and character of metastases found at postmortem examination, it is likely that a number would ultimately have died from the effects of the tumor. In some of these animals, a considerable number of metastatic foci were found while in others, there was comparatively little tumor but this involved such structures as both suprarenal glands or the bones of the jaw or the cranial sinuses and adjacent bones. The number of so called probable deaths was lowest in the controls and highest among the dark groups as shown by the following figures:

	No of rabbits	No of actual deaths	No of probable deaths	Incidence of probable deaths	
				Relative rate	Actual rate
				per cent	*per cent*
Controls......................	37	8	2	5.4	6.9
Darks	38	7	9	23.7	29 0
Lights.......	36	4	4	11.1	12.5

The actual death rate was, as shown above, slightly higher in the control than in the dark room groups, but if the number of probable deaths is also considered, then the highest total mortality occurred in the latter groups. The lowest rates from both standpoints occurred in the groups exposed to constant light.

The character of the disease in the instances of probable deaths will be discussed later in connection with the distribution of metastases, but it may be said now that the outstanding feature of these cases was the high incidence and number of secondary growths in the bones.   This is of special interest in connection with the proportion of actual deaths directly caused by bone involvement and here again the dark group showed by far the highest incidence.

	Actual deaths	Fatal metastases to bone		
		No.	Rate	Location
			per cent	
Controls	8	3	37.8	All spine
Darks	7	5	71.4	4 spine
				1 jaw
Lights	4	2	50.0	All spine

Postmortem Observations of Metastatic Foci.—Perhaps the most indicative single feature of the tumor process for evaluating the general character of the disease is the distribution and state of secondary growths.

The metastatic foci found at postmortem examination have been analyzed, first, from the point of view of their incidence and total number and, second, from their distribution to certain organs and tissues.   As is shown in Table III, the incidence of secondary growths was practically the same in the control and dark groups of 2 experiments, I and II, but it was considerably lower in the case of the light group, while in the 3rd and 4th experiments, it was practically the same in all 3 groups.   If the 4 experiments be combined, it is seen from the following figures that there was little difference in the metastatic incidence for the controls and the rabbits from the dark room, but that the rate for the light groups was considerably smaller.

	No. of rabbits	No. with metastases	Incidence
			per cent
Controls	37	21	56.75
Darks	38	23	60.52
Lights	36	14	38.88

The total number of sites involved by secondary growths in the control groups varied greatly, that is, from 6 to 74, and there was much the same variation among the dark groups, that is 13 to 69. In 2 experiments, I and IV, there were slightly fewer foci among the groups from the dark room than among the controls, in No. III, there was the same number, but in the 2nd experiment, there were more in the dark group due to a single instance of a comparatively severe

TABLE III.

Analysis of Metastatic Foci as Observed at Postmortem Examination.

Experiment No.	Group	No. of animals	Incidence No.	Incidence Per cent	Total No.	Rate Relative	Rate Actual	No. of foci in Deaths	No. of foci in Survivors	
I	C.	11	9	81.9	74	6.7	8.2	26, 19, 14, 6*	4, 2, 1, 1, 1	0 in 2 rabbits.
	D.	12	9	75.0	69	5.8	7.7	19, 6, 2,	24, 6, 5, 5, 1, 1	0 " 3 "
	L.	12	4	33.3	37	3.1	9.3	4	18, 13, 2	0 " 8 "
II	C.	10	3	30.0	6	0.6	2.0	——	3, 2, 1	0 " 7 "
	D.	9	3	33.3	13	1.4	4.3	10	2, 1	0 " 6 "
	L.	10	1	10.0	3	0.3	3.0	——	3	0 " 9 "
III	C.	11	7	63.6	58	5.3	8.3	24, 15, 14	2, 1, 1, 1	0 " 4 "
	D.	12	9	75.0	58	4.8	6.4	12, 6	19, 7, 5, 4, 2, 2, 1	0 " 3 "
	L.	10	7	70.0	44	4.4	6.3	20, 9, 7	3, 3, 1, 1	0 " 3 "
IV	C.	5	2	40 0	34	6.8	17.0	28	6	0 " 3 "
	D.	5	2	40 0	15	3.0	7.5	13	2	0 " 3 "
	L.	4	2	50 0	8	2.0	4.0	——	7, 1	0 " 2 "

* Complicating empyema.

disease in an entire series of very low malignancy. This case was probably a purely chance occurrence which might have been included in either of the other groups, but it is perhaps significant that it did not occur in the light group. The variation in the numbers of metastatic foci was not as marked in the light groups—3 to 44, and there were fewer foci in all light groups than in the corresponding controls. This difference amounted to 50.0 per cent in Experiments I and II, to 24.0 per cent in No. III and to 76.0 per cent in No. IV, in terms of the control values.

The relative rate of metastases, that is the number of foci per rabbit, was uniformly the lowest in the case of all light groups while the rates for the dark groups were somewhat lower than those of the controls except in Experiment II. The value of the relative as contrasted with the actual rate in which only the animals with secondary growths are considered, lies in the fact that the latter is accentuated by the inclusion of 1 or 2 rabbits with numerous metastases. Both rates, however, are useful in furnishing information on the extent of variation in tumor distribution. In these experiments, the control groups were characterized by the most irregular distribution while the groups from the dark room were the most uniform, as shown by the following figures:

Metastatic foci		Controls	Darks	Lights
Relative rate	I........	6.7	5.8	3.1
	II........	0.6	1.4	0.3
	III........	5.3	4.8	4.4
	IV........	6.8	3.0	2.0
Actual rate	I........	8.2	7.7	9.3
	II........	2.0	4.3	3.0
	III........	8.3	6.4	6.3
	IV........	17.0	7.5	4.0

If the numbers of foci in the different experiments be combined, the greatest number together with the highest relative and actual rates is found to have occurred in the controls.

	Total No. of foci	Relative rate	Actual rate
Controls....................................	172	4.65	8.18
Darks....................................	155	3.89	6.43
Lights....................................	92	2.55	6.56

As compared with the controls, the distribution of secondary growths was somewhat more restricted among the rabbits from the dark room and markedly so in the animals exposed to constant illumination. On the other hand, it would appear from these combined values that as far as the actual rate is concerned, tumor dis-

tribution was as well marked in the light as in the dark series, a fact which alone might suggest comparable degrees of disease severity. That such was not the case has already been shown by such indices as the mortality rate, the incidence of metastases and the total number of metastatic foci, and an additional demonstration is afforded by an analysis of the character of metastatic foci from the standpoint of the organs and tissues involved.

TABLE IV.

Summation Values of Metastatic Foci.

Experiment No.	Group	Suprarenals and eyes	Extensions and implantations	Lungs and pleura, liver, kidneys, pancreas	Skin, subcutaneous tissue, superficial lymph nodes, bones, muscles, endocrine glands, spleen, central nervous system
		per cent	*per cent*	*per cent*	*per cent*
I	C.	250.0	121.2	200.0	103.1
	D.	275.0	136.7	120.0	86.3
	L.	75.0	89.5	120.0	36.7
II	C.	25.0	10.6	60.0	0
	D.	100.0	10.6	60.0	13.2
	L.	0	5.3	40.0	0
III	C.	75.0	105.3	220.0	82.0
	D.	225.0	110.6	140.0	69.8
	L.	275.0	63.3	120.0	26.7
IV	C.	50.0	63.1	80.0	53.3
	D.	125.0	21.1	40.0	13.3
	L.	0	21.1	40.0	6.7

Character of Metastic Involvement.—The method used in determining the relative severity of the disease from the particular organs and tissues involved has already been described in the section on Materials and Methods.

In Text-fig. 1, the summation values of metastatic foci of the dark and light groups are contrasted against those of the controls (Table IV). The most conspicuous feature of this chart are the 4 columns above the base line, representing the greater distribution of metastases to the suprarenals and eyes in 3 dark and 1 light group (Experiments
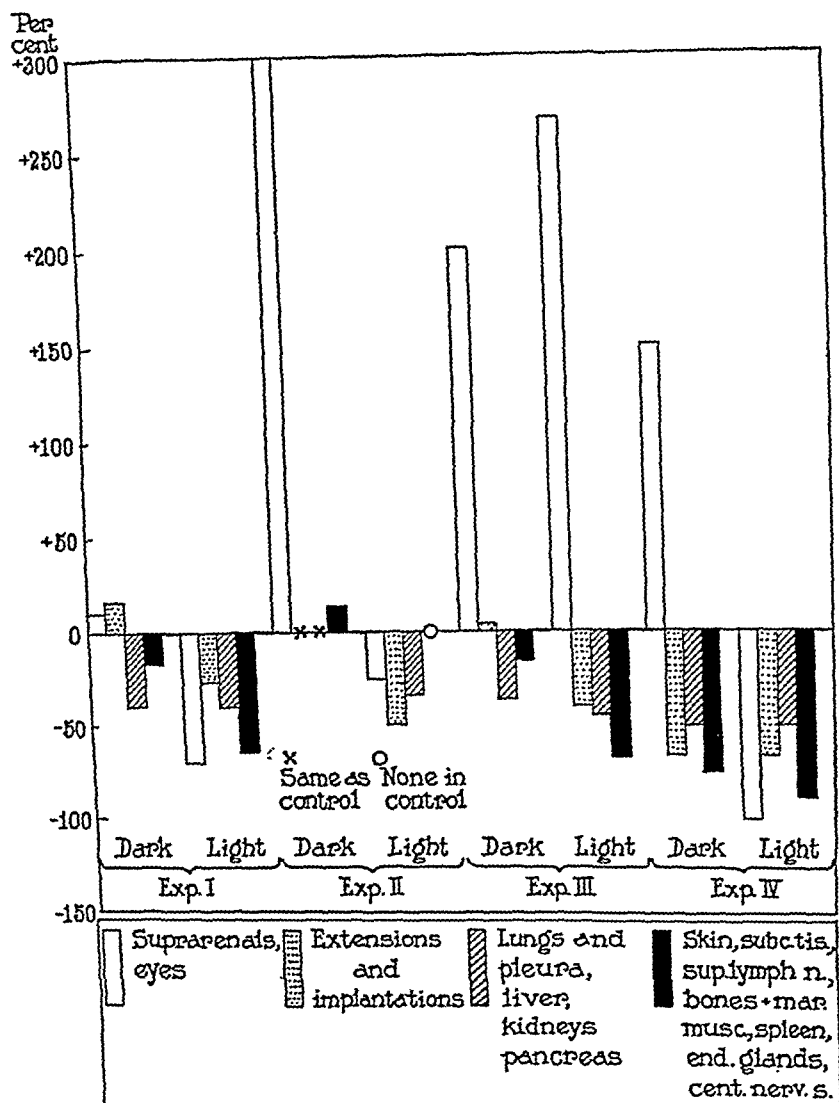
TEXT-FIG. 1. Summation values of metastatic foci in individual experiments expressed in percentage variations of control figures.

II, III and IV). The disproportionate length of these columns as compared with the others is due, first, to the fact that there are only 4 possible sites for secondary growths in this division as contrasted with 5, 19 and 30 in the others, and, second, to the relative frequency of metastases to these organs in cases of low as well as of high malignancy. In so far as severity of the disease is concerned, however, secondary growths in the suprarenals and eyes are of relative unimportance as compared with those to the viscera, other glands of internal secretion, muscles, bones and skin, which in this chart are represented by the last 2 of each set of 4 columns. In the case of the rabbits exposed to constant light, the distribution of metastases to these significant sites of possible tumor growths was, in each experiment, considerably less marked than it was with the controls as indicated by the length of the columns *below* the base line. A further indication of the comparatively limited distribution of metastases in the light groups is brought out by the position of the first 2 columns representing extensions and implantations to the deep lymph nodes and serous membranes and growths in the suprarenals and eyes. These columns are in all but the one instance mentioned above (Experiment III) *below* the base line.

As far as the groups from the dark room are concerned, reference to the chart shows that in 3 experiments, Nos. I, III and IV, metastases to the viscera, endocrine glands, muscles, bones and skin were not as widely distributed as they were in the control groups, but that the difference was not as marked as it was in the case of the rabbits from the light room. In Experiment II, the distribution of tumor in the divisions of extensions and implantations and of the viscera was the same as in the controls, but was slightly greater in the case of the division of skin, bones, etc. This exception, as has already been mentioned, is due to the occurrence of 1 instance of a comparatively severe disease in a series of low malignancy.

The last chart (Text-fig. 2) in which the summation values for metastatic foci of the 4 experiments have been combined illustrates the outstanding features of the distribution of secondary growths in the rabbits exposed to constant illumination or to constant darkness, as compared with those kept under indoor conditions of variable diffused sunlight. In the case of the animals from the light room,

there was a much more limited distribution of tumor in all 4 divisions
of metastatic foci, especially in the last division which is particularly
involved in instances of pronounced malignancy.   In the case of the
rabbits from the dark room, secondary growths were also less widely
distributed to 3 divisions of possible tumor foci, but were more fre-
quent in the division of the suprarenals and eyes.   As compared with
the light groups, the reduction of summation values was not as
pronounced.
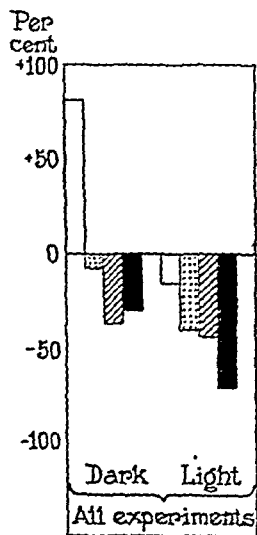
TEXT-FIG. 2. Combined summation values of metastatic foci expressed in
percentage variations of control figures.

In discussing the mortality rate, mention was made of the prob-
ability that if certain rabbits had been allowed to live beyond the
experimental period of 2 months, they would ultimately have died
from the effects of tumor growth and statistics were given which
showed that the largest number of these cases was found in the groups
from the dark room. This aspect of the disease deserves further
discussion and may conveniently be considered at this point in con-
nection with the general character of metastatic distribution.

There were 4 instances equally divided between the dark and light

groups of relatively malignant but slowly progressive tumors (Table III). Postmortem examination 2 months after inoculation revealed numerous foci of secondary growth which would certainly have brought about a fatal termination had the animals been allowed to live beyond the experimental period. There were no control rabbits with this type of disease. No difficulty is experienced in recognizing such cases of probable deaths, but there were 11 other rabbits which have been similarly classed because of the location and state of the metastases, although these were comparatively few in number. There were 7 such instances in the dark, 2 in the control and 2 in the light groups, respectively.

The most frequent sites of secondary growths in this type of presumably fatal disease are the suprarenal glands, but in these experiments, there was also an unusual number of metastases to the bones of the face and jaws and the cranial sinuses, particularly in the rabbits from the dark room as shown by the following combined group figures:

	Probable deaths omitting malignant cases	Total foci of metastases	Foci of bone metastases
Darks	7	27	6 or 22.2 per cent
Lights	2	10	1 " 10.0 " "
Controls........................	2	9	0 " 0 " "

These findings are in line with the greater incidence of bone metastases in the dark groups than in either the control or light series. The observations of individual experiments, as well as the combined values are given below to show that this condition was present in each experiment.

Incidence of Metastases to Bone.

	Controls	Darks	Lights
	No. of rabbits	No. of rabbits	No of rabbits
Experiment I	2 or 18 2 per cent	7 or 58 3 per cent	2 or 16.7 per cent
" II	0 ————	1 " 11.1 " "	0 ————
" III	3 " 27 3 " "	5 " 41.7 " "	3 " 30 0 " "
" IV	1 " 20 0 " "	2 " 40.0 " "	1 " 25.0 " "
Total...............	6 " 16.2 " "	15 " 39.4 " "	6 " 16.7 " "

It will be recalled that in discussing the general character of the disease in the control groups, it was pointed out that the incidence of bone lesions was considerably greater than in the first 20 generations of the tumor. The above data show that in respect to the incidence of this type of metastasis the groups from the light room did not differ from the controls, but that there was a much higher rate among the dark groups. The environment of constant darkness evidently favored the increased tendency toward bone lesions prevailing at this time.

The foregoing analysis of clinical and postmortem data may be briefly summarized at this point before discussing the significance of the results obtained. The tumor process in the control groups exposed to variable diffused sunlight varied from a very mild condition in 1 experiment to a considerably more severe disease in 2 others, but in none of the 4 was a level of well marked malignancy attained. It was much more severe, however, than the disease which developed in the groups exposed to constant illumination and somewhat more so than in the groups kept in constant darkness. As compared with the controls the light groups were characterized by the following features:—an unusual number of failures to obtain a primary growth, less active and less extensive primary tumors, a lower mortality rate, a lower incidence and a smaller number of metastases recognizable during life and at postmortem examination and a much greater restriction in the distribution of these growths. It is significant, moreover, that the influence of constant illumination in the direction of diminished malignancy was observed at times of mild as well as of more severe disease.

A similar comparison of dark groups showed that the growth of the primary tumors was more irregular but on the whole not as pronounced or as persistent as in the controls. On the other hand, the mortality rates and the incidence of metastases found during life and at autopsy were only slightly lower than control values. The number of metastases, however, was smaller, the relative and actual rates were lower and the distribution of these growths was definitely more restricted. An interesting aspect of the tumor process in these rabbits was the tendency toward group uniformity in regard to certain features of the disease. Thus, in the 4 experiments the dark groups as compared

with the control or the light series, showed less variation in the incidence of secondary growths recognizable during life, in the mortality rates and in the relative and actual rates of metastatic distribution. A similar tendency has also been observed in the dark groups of experiments with *Treponema pallidum* (7). Although the influence of constant darkness was exerted toward diminishing tumor malignancy, it was much less pronounced than that of constant illumination, and, furthermore, it had little or no effect in the series in which the disease was of a mild character. It was also modified by the peculiar nature of the tumor process of the 4 experiments, which was characterized by a high incidence of bone metastases despite the moderate or low level of malignancy that prevailed. Conditions of constant darkness were associated with a greatly increased incidence of these lesions which, due to their location in such sites as the spinal column and jaws, were directly responsible for a large proportion of actual as well as of probable deaths.

The results of a previous experiment (1) with constant illumination (Mazda lamps and mercury arcs in crown glass) and constant darkness were of the same order of diminished malignancy but more striking. This was especially true of the group from the light room and while the difference in the case of the dark group was not pronounced, it was definitely greater than in the current experiments. The present analysis would tend to show that the greater severity of the tumor process prevailing at the time of the previous experiment undoubtedly was 1 factor that influenced the particular character of the results obtained. The 2 types of illumination must also be considered, and from this standpoint the combination of Mazda lamps with mercury arcs was more effective than the arcs alone, if one may judge from the single experiment in which the former system was used.

It is obvious that before one can ascribe the effect of diminished malignancy solely to the influence of constant illumination or of constant darkness, other possible factors must be considered. The state or condition of the animal organism prior to exposure to these surroundings is probably of considerable significance and it is conceivable that different states of the host might react in a different manner to these conditions of light and darkness with materially

dissimilar results. Indeed, our explanation of the variations in tumor malignancy is largely based upon the idea that resistant or susceptible states of the host are dependent upon different conditions of animal economy, which may be affected by innumerable factors.

One must also take into account the fact that in these experiments, the animals were subjected to the influence of the factor of change incident to their removal from the variable diffused sunlight of the laboratory to constant conditions of continuous light or of continuous darkness. Little is known of the importance of this factor in relation to the reaction of animals to experimentally induced disease but there are certain observations with this tumor which suggest that the effect of change in external conditions may manifest itself in the direction of increased malignancy. For instance, in 1 experiment dealing with this question, the tumor process in a group of 10 rabbits recently brought to the laboratory was much more severe than in a similar sized group assembled 2 months prior to inoculation.

In order, therefore, that the influence of such a physical force as constant illumination on tumor malignancy be fully effective, it is probable that some sort of initial adjustment or accommodation to the new environment must take place, as experiments begun before its completion would necessarily be complicated by the process whatever it may be. The length of time required for such an adjustment under the conditions of these experiments is not known, but previous work dealing with the changes in organ weights of normal rabbits under similar environmental surroundings, suggested that a 4 weeks' period was associated with relatively stable weights of most organs (6). Consequently, in the majority of tumor experiments, inoculations have been carried out after 4 weeks' exposure to the light or dark conditions. In this connection, the results of an inoculation after 2 weeks' exposure are of interest. The group from the light room showed a less severe disease than the controls, but in the group from the dark room, the tumor process was distinctly more malignant. While the observations of a single experiment are not conclusive, the results of the dark group are in line with what might be expected if the 2 weeks period was not sufficient for the completion of adjustment to this condition. Under these circumstances, the effect of the factor of change would still be operative and if its influence in the

direction of increased malignancy were sufficient, one would expect a more severe disease. On the other hand, a 2 weeks' period was evidently sufficient as far as constant illumination was concerned for the establishment of an adjustment of the animal organism to this condition, or, and this may prove to be the correct explanation, the effect of the light might have been such as to mask or effectually modify the influence of other factors operating in the direction of increased malignancy.

The results of these experiments are interpreted as furnishing evidence in support of the belief referred to in the beginning of this paper, that there is a relation between the factor of light and the manifestations and course of the disease induced by a transplantable malignant neoplasm. The mechanism by which the effects are produced is not known. We have considered the influence of the seasonal factor in this condition and in experimental infections of the rabbit induced by *Treponema pallidum* as operating upon or affecting animal economy and susceptibility or resistance as a functional activity of the animal organism. From this standpoint, it appears that resistance to tumor growth as displayed by rabbits living under certain conditions of constant illumination and to a less extent, in almost constant darkness, was more pronounced than it was in the case of control rabbits exposed to the daily fluctuations of diffused sunlight.

SUMMARY.

Experiments are reported in which an environment of constant and continuous light with a wave-length of from 3022 to 5790 Ångstrom units, supplied by mercury arcs in crown glass, and of constant darkness, have influenced the course and character of a malignant disease of rabbits induced by a transplantable neoplasm.

Under the influence of constant light the level of malignancy was lower than in control animals living under ordinary conditions of diffused sunlight. Under the influence of constant darkness the level of malignancy was somewhat lower than in control animals but the effect of this environment was modified by the special character of the disease prevailing at this time. The incidence of bone metastases was unusually high, but it was greatly increased in the rabbits kept in constant darkness.

These observations furnish experimental evidence in support of the belief that there is a correlation between the external factor of light and the manifestations of an experimental malignant disease.

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EXPERIMENTAL MEDICINE

EXPERIMENTAL BONE MARROW REACTIONS.

III. POLYCYTHEMIA, NORMOBLASTOSIS, AND ERYTHROCYTIC HYPERPLASIA OF THE BONE MARROW PRODUCED BY GUM SHELLAC.

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PLATE 26.

In the study, both experimental and clinical, of the hematopoietic system, attention has been directed almost exclusively to the peripheral blood. This is due to the ease with which the peripheral blood may be examined and the difficulties encountered when attempts are made to analyze the bone marrow and interpret the results obtained. The recent work of Sabin, Doan, and Cunningham (1–3), however, on the structure and function of the bone marrow has greatly facilitated the study of the hematopoietic organs. The theory of these authors as to the origin of red blood cells and clasmatocytes from the endothelium lining the sinuses of the bone marrow, furnishes a working hypothesis for the study of the function of the bone marrow, and its reaction to external influences.

In a series of investigations on bone marrow reactions the hypothesis of the endothelial origin of the red blood cells and the clasmatocytes has been used as a working basis. In a former study (4, *a*) it was found that a colloidal suspension of a heavy metal (collargol) produced an aplastic anemia, interpreted as due to injury to the endothelial cells of the hematopoietic organs. In a control experiment (4,*b*) with a colloidal suspension of carbon particles in the form of drawing ink, the results warranted the conclusion that carbon particles as such stimulate phagocytosis, but do not inhibit, to any appreciable extent, the formation of erythrocytes. This seems to indicate that the particles ingested by the phagocytic cells must exert a toxic action if they

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are to suppress the function of the cells. The importance of this is evident in the choice of colloids for so called "blocking" of the "reticulo-endothelial system."

During the latter investigation with India ink, a non-dialysable substance passing through a Berkefeld filter was found to stimulate markedly the bone marrow as evidenced by the outpouring of nucleated red cells in the peripheral blood and erythropoietic hyperplasia of the bone marrow. Through the courtesy of F. Weber Co. the composition of this particular ink was obtained. All the ingredients were tried, but only one gave the results interpreted as a stimulation of the bone marrow. This was a gum shellac used as a protective colloid. Experiments were continued with this substance with two purposes in mind: first, to elucidate, if possible, the mechanism of stimulation of the red cells and their delivery, and secondly to see whether this gum would prove to be a therapeutic agent. The second project, in view of the many substances proposed and rejected as bone marrow stimulants, was entered upon with justifiable hesitation.

Most of the work on stimulation of the erythropoietic centers has been done in search for a therapeutic agent, the importance of which is evident and reflected in a vast literature.

No attempt will be made to review this as recent papers have summarized different phases of the question (5, 6, 7, a, 8). If, however, one accepts as criterion of erythropoietic activity the rise of the number of erythrocytes, together with the appearance of young cells in the peripheral blood stream and a hyperplastic marrow, the experimental evidences are meager. Polycythemia, due to various agents, is common. Many of those reviewed by Lamson (5) in 1915, however, may be explained in all probability by other conditions than actual bone marrow stimulation. Others give data insufficient for drawing any definite conclusions.

The recent search for therapeutic agents, stimulated by advances in our knowledge of the internal secretions and hormone activities, has been directed toward the isolation of substances in the animal organism. Following Danilewsky and Selensky (9), Krumbhaar and Musser (10) and Downs and Eddy (11) found that spleen extract caused a rise in the number of red blood cells with comparatively less increase in hemoglobin. Krumbhaar and Musser obtained no evidence of an increased number of young cells in the blood, while Eddy and Downs (7,b) found a gradually increasing normoblastosis in rabbits given large doses of protein-free splenic extract subcutaneously. The number of red cells in the peripheral blood fluctuated with a generally rising tendency. Neither of these authors report any examination of the bone marrow.

Leake and associates (12, *a*, *b*) found a combination of splenic and bone marrow extract more effective than splenic extract alone. In the rabbits injected the reticulated cells showed an increase in a few cases, while of six animals examined the bone marrow in two was hyperplastic. Whether this hyperplasia was erythropoietic is not stated.

The reports of Eddy and Downs (7, *b*) and Leake and Leake (12, *a*) seem to indicate that some definite effect was produced on the bone marrow. They conclude that hormones are involved. That some substance capable of stimulating red blood cell formation is present in the serum and the bone marrow after bleeding was suggested by Carnot and Deflandre (13).

Among inorganic agents for stimulation of the erythropoietic organs, iron and arsenic have been used extensively in clinical work. Experimental work seems to indicate that neither iron nor arsenic exerts any marked effect on blood formation (14–16). Nissen (17), however, reports that erythroblastic hyperplasia is produced by the intravenous injection of "elektroferrol." Excluding anemia from various causes, and especially anemia due to blood loss, low oxygen tension (18, 19) seems to be the most effective stimulant of blood formation as evidenced by increase in erythrocytes and a hyperplastic marrow.

Material and Methods.

The gum shellac employed was obtained from the F. Weber Co., Philadelphia. Gum shellac is said to be a product of plant excretion or secretion, due to the action of the lac insect, *Coccus lacca*. Chemically it has been partly analyzed although some of its components are very unstable (20). The gum shellac used is soluble in water containing sodium borate. In concentrated solutions it is a cloudy dark brown fluid which in dilutions employed after filtering appears as a clear, port wine-colored liquid. The solutions employed varied in strength from 4.4 to 0.44 per cent of the gum, and, correspondingly, the salt used to keep the gum in solution varied from 1.8 to 0.18 per cent. The solutions were sterilized in the autoclave, occasionally by boiling. Injections varied both in amount and interval of administration, as it was desirable to see the effects not only of various concentrations but also of the frequency of administration. The experimental procedure was essentially the same as in a previous investigation (4, *b*).

Bone marrows were studied from three points of view: in sections, in smears made directly from the bone marrow, and in smears made after centrifuging the emulsified bone marrow in 2 per cent sodium citrate. In addition, supravital counts were made of all the bone marrows, following the method of Sabin (3, *b*).

Rabbits bought in the market usually improve when given better food in the laboratory. To exclude this factor as far as possible, they were kept in the stock pen for a considerable period, then put in individual cages under the same conditions for from 2 to 4 weeks before the investigation was begun.

EXPERIMENTAL.

Healthy male rabbits were used except in a few instances which will be noted. They were divided into groups according to the strength of the solution employed: (1) Strong solution—equal to the amount of gum shellac contained in the original ink; *i.e.*, 4.4 per cent. This was intensely toxic. (2) Medium solution—2.2 per cent. This was toxic to some animals, stimulating to others, as evidenced by increase of red blood cells and hemoglobin and a characteristic outpouring of nucleated red cells. (3) Weak solution—1.1 per cent. This was usually stimulating, although slightly so in some cases. In 0.4 per cent solutions no reaction was elicited.

I. Injections of 4.4 Per Cent Gum Shellac Solution.

Nine animals were injected with a 4.4 per cent solution. Of these five died instantaneously after the first intravenous injection of 4 cc., two died suddenly after the second dose, and one after four doses of 2.5 cc., and a fifth dose of 3 cc. The remaining animal had seven doses of 4 cc., subcutaneously, without apparent systemic effects. Locally there was induration and edema of the subcutaneous tissues but no apparent necrosis.

The mode of death in the animals was similar. About $\frac{1}{2}$ minute after the injection, and in a few instances before the needle had been withdrawn, there were signs of collapse with rapid breathing but little or no muscular contraction of body and limbs. Then the breathing stopped while the heart continued to beat for a short varying interval after the cessation of respiration. At autopsy the lungs were found collapsed with little or no injection; the right heart was distended and engorged with blood; the left heart was empty; the abdominal viscera were greatly congested. The impression was gained that there was a sudden interruption of the blood flow in the pulmonary circulation. The microscopical sections gave no clue to the cause of death. Many of the smaller and medium sized vessels were closely packed with erythrocytes but no agglutination of the latter had taken place as far as could be determined from fixed sections.

In conjunction with Dr. V. H. Norris, the effect of the solution on blood pressure and respiration was tested. With a fatal dose in an

animal under heavy morphine anesthesia the respiratory rate increased and the blood pressure rose, but the animal had convulsions in the agonal struggle thus interrupting the record. When smaller, non-fatal doses were given, there was a definite increase in the respiratory rate as well as a rise in blood pressure, both well sustained for a considerable period of time. Physiological sodium chloride in the same dosage had no effect on respiration or blood pressure.

The cause of sudden death in animals from toxic substances as well as death due to anaphylactoid phenomena has been analyzed by many observers (21–23). It is probable that the fatal outcome in the animals injected with 4.4 per cent gum solution may be explained as an anaphylactoid phenomenon, although paralysis of the respiratory center cannot be excluded. The fact stands out that a solution of this strength given intravenously was fatally toxic to all the animals injected. This is in sharp contrast to the effect of the same substance in contact with carbon particles in the India ink which in no instance was fatal in the same concentration. The inert carbon particles must have exerted a detoxifying influence on the solution in which they were suspended. That they acted as a protective was shown in one animal which had been injected with India ink to which had been added an equal quantity of the 4.4 per cent solution of gum shellac. Three injections of 4 cc. each were given without ill effect. The fourth injection of 4 cc. of the solution in which the ink had been replaced by distilled water caused the death of the animal in the same sudden manner as described above.

II. Injection of 2.2 Per Cent Gum Shellac Solution.

Seventeen rabbits were injected with 2.2 per cent gum shellac solution. Of these four died as a result of the injection. Three died suddenly, one after the first, one after the second, and one after the fifth dose. The remaining animal was found dead 24 hours after the second dose.

Microscopically, No. H-76, succumbing after the first dose, showed extensive hemorrhage in the bone marrow, amounting practically to a dissolution of the vascular bed. This animal had indications of a hyperplastic marrow prior to the injection caused by loss of blood. No. H-91, dying after the fifth injection, showed a hyperplastic bone marrow, comprising mainly the erythrocytic series, with a

moderate number of megaloblasts and erythroblasts and a great many normoblasts. Endothelium, wherever seen, was hypertrophied. In addition, the megalocaryocytes were increased and an occasional clasmatocyte was seen filled with a yellowish pink substance which did not take the iron stain. No pathology to account for the sudden death could be made out in any of the animals except in No. H-95 which died 24 hours after the second dose, showing fibrinous exudate in the lung alveoli.

Thirteen rabbits were repeatedly injected with a 2.2 per cent gum shellac solution. All these animals showed one thing in common; namely, the outpouring of normoblasts and later erythroblasts in the
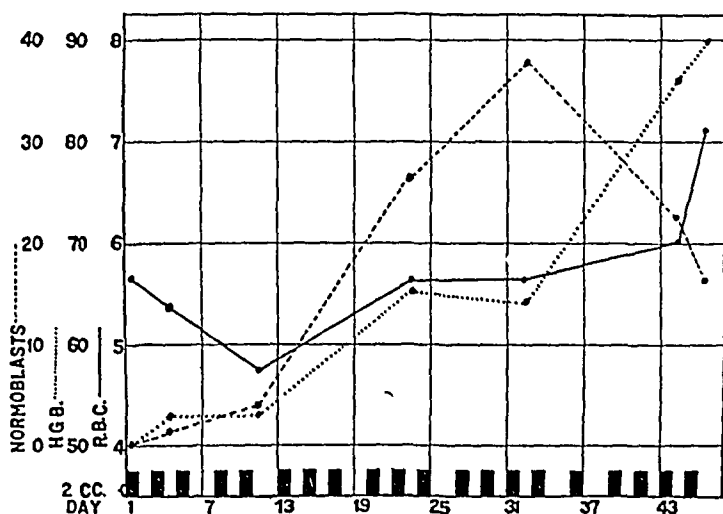
In Charts 1 to 5 the number of normoblasts is in thousands, the hemoglobin in per cent, and the erythrocytes in millions.

CHART 1. Rabbit H-83 illustrates the rise of hemoglobin and erythrocytes and the outpouring of normoblasts after medium sized doses in an animal with achromia of the red blood cells. The bone marrow showed intense hyperplasia.

peripheral blood. The number of red blood cells and the hemoglobin values following the injection of this strength solution were variable, some animals showing a slight initial drop after several doses, followed by a rise, others an initial rise followed by a decrease. The lowest point of erythrocytes and hemoglobin was usually concomitant with the greater number of nucleated red blood cells in the peripheral blood stream, although exceptions occurred.

Rabbit H-83, Chart 1, when injections were begun, was in good condition except for low hemoglobin and a marked achromia of the erythrocytes. Twenty 4 cc. injections were administered over a period of 45 days. The first few doses were followed by a slight fall in the number of erythrocytes, concomitant with a slight increase in white blood cells; then there was a definite rise, both in hemoglobin and erythrocytes. Nucleated red blood cells were present in the peripheral blood after the second dose, the highest number (37,600 per c.mm.) being reached on the 32nd day, after which there was a decline with still further increase in hemoglobin and erythrocytes. The animal was killed at a time when the hemoglobin showed an increase of 80 per cent of the initial determination and the red cells an increase of approximately 36 per cent. The general condition was excellent, the weight had
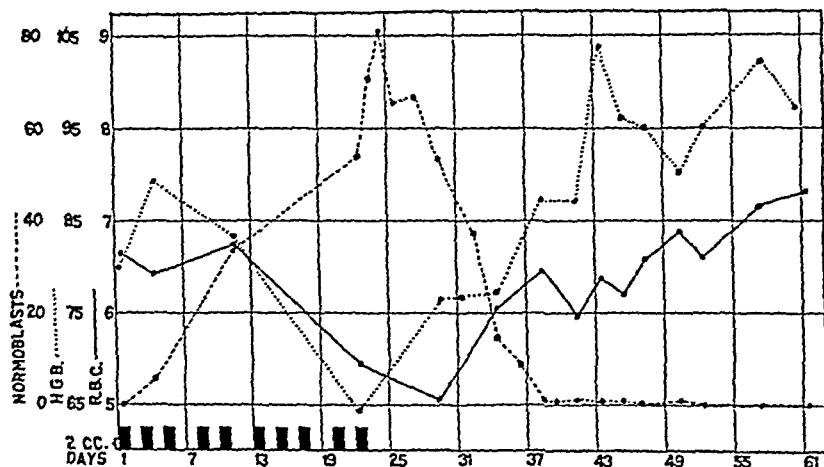
CHART 2. Rabbit H-84 showed a tremendous number of nucleated red cells in the peripheral blood stream, which did not disappear until the 28th day after the last injection. Bone marrow 38 days after last dose was essentially normal.

increased from 2610 to 2775 gm. The gross autopsy findings were normal except for an enlarged spleen and liver. Microscopically, the liver, spleen, and bone marrow showed a great many phagocytic cells, enlarged, staining a pale pink with eosin, and containing various sized conglomerations of a substance staining a deep red. The spleen contained many nucleated red cells, mainly normoblasts. Many open spaces were lined with cells which looked like erythroblasts and megaloblasts, and the impression was gained that erythrocyte hyperplasia was present in the spleen. The protoplasm of the liver cells looked vacuolated and granular, but as this was observed in untreated animals it probably may be disregarded. The kidney showed a few patches of fibrosis in the medullary region with occasional round cell infiltration in the cortex. There was no indication of increased blood destruction, no iron being found. The bone marrow was intensely hyperplastic.

This hyperplasia included mainly the erythrocyte series and the megalocaryo-cytes. The normoblasts and late erythroblasts predominated but a consider-able number of the earlier forms were also present (see Fig. 1.) The predominance of the nucleated red cells was verified additionally by (1) supravital studies, (2) fixed smears prepared directly from the bone narrow, and (3) fixed smears pre-pared after centrifuging the emulsified bone marrow in 2 per cent sodium citrate. From these additional studies it was found that the leucocytic elements consti-tuted about 25 to 38 per cent and the nucleated red cells from 62 to 75 per cent, varying with the method used, but giving an average value of one-third white blood cells and two-thirds nucleated red cells. That the increase was considerable may be concluded from the fact that most of the fat cells of the bone marrow had been replaced, although some deduction of space must be made for the clasmato-
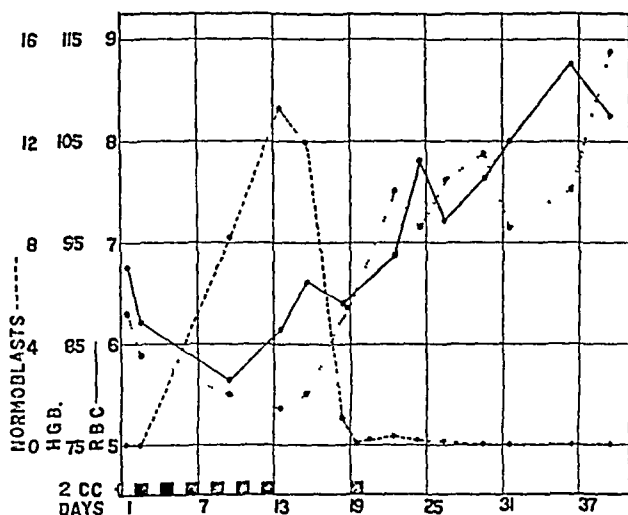
CHART 3. Rabbit H-100 illustrates the effect of small dosage. Normoblastosis was less striking, but the final rise in erythrocytes and hemoglobin was marked.

cytes which were both enlarged and increased in number. The blood vessels of the bone marrow were everywhere intact but the high power lens frequently showed these vessels to be lined with nucleated red cells in various stages of maturation (see Fig. 2)

To see how long the presence of nucleated red blood cells would continue in the peripheral blood, Rabbit H-84 was given 10 doses of a 2.2 per cent solution (see Chart 2) On the 2nd day after the last dose 82,400 erythroblasts and normo-blasts per c.mm. were found in the blood stream. Not until the 28th day after the last injection did the nucleated red blood cells disappear permanently from the peripheral blood stream. The stimulation in this animal seemed to produce a tremendous excess of cells of the erythrocyte series, and the subsequent course indicated that these cells gradually matured and were thrown into the peripheral

circulation, increasing the hemoglobin and the red cells. At autopsy, 38 days after the last dose, little or no evidence was present in the bone marrow to indicate the stage of intense erythropoietic hyperplasia through which the animal had passed.

Rabbit H-100, Chart 3, illustrates the effect of smaller dosage (2 cc.). The initial decrease of red blood cells and hemoglobin was negligible, the outpouring of the nucleated red cells was correspondingly less, the final rise of hemoglobin and erythrocytes was marked, with values customarily not encountered in rabbits. There was an increase in weight from 2755 to 3160 gm., and when killed the animal was in perfect condition except for an enlarged but normal looking liver and kidney. Microscopically, the bone marrow was normal except for slight irregularity in the contour of the fat cells. By estimation the proportion of cells seemed normal; *i.e.*, about two-thirds leucocytes, one-third of the erythrocyte series. This was verified by fixed smears and supravital studies in which the actual counts varied from 33 to 37 per cent nucleated red blood cells, and from 63 to 69 per cent white blood cells. A few clasmatocytes were enlarged in the bone marrow; the number of megalocaryocytes was not increased.

Another animal treated similarly to No. H-100 showed the same results somewhat less marked.

In control animals the cells in the bone marrow averaged one-third nucleated erythrocytes and two-thirds leucocytes. This is in agreement with Itami (24) who found that the nucleated red bood cells in the normal rabbit's marrow constituted $29\frac{1}{2}$ per cent, excluding primitive cells. In comparing animals which were killed immediately after discontinuation of the injections with animals allowed to live for a variable time, the impression was gained that the enlarged clasmatocytes had decreased in number in those allowed to live, thus indicating that an elimination of their contents was taking place.

III. Injection of 1.1 Per Cent Gum Shellac Solution.

Twelve rabbits were injected with 1.1 per cent solution. Both dosage and time interval between injections were varied to see whether optimum effects could be obtained.

With 4 cc. doses given at the same intervals as No. H-100, Chart 3, the results were practically the same as with 2 cc. of a 2.2 per cent solution.

The effect of 4 cc. doses administered at longer intervals is illustrated in Chart 4, Rabbit H-104. At the beginning of the investigation this animal was thin, ill

looking, anemic, with 2,970,000 red blood cells and 52 per cent hemoglobin. No cause for the anemia was discovered; the animal had been isolated for some time and no external injury or blood loss could be found. The peripheral blood findings remained stationary for about 1 week except for the day on which the first injection was made when the reticulated count had risen to 9.5 per cent. The response was prompt. 3 hours after the injection the reticulated count rose to 15.3 per cent, with 1200 nucleated red blood cells per c.mm. Nucleated red blood cells were present in the blood stream up to the 4th day as a result of the first injection. Another injection 4 days after the first dose caused a still greater outpouring of normoblasts. At this time the reticulated count was still high; namely, 13.2 per cent. Before the third injection was given all the normoblasts
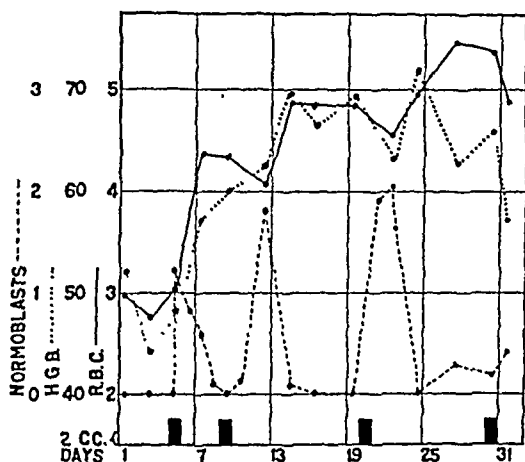
CHART 4. Rabbit H-104. Thin, anemic animal given 4 cc. doses of 1.1 per cent gum shellac solution at long intervals. The prompt response to each injection is noteworthy. Final hemorrhages in the glomeruli of the kidneys.

had disappeared from the peripheral blood and the reticulated count was approximately normal (2.6 per cent). The prompt response to the third injection is indicated on the chart. In 26 days the blood count had reached a low normal value and the animal was gaining weight, although far from appearing healthy. The fourth injection was given 10 days later. Next morning the rabbit was moribund. At autopsy the large kidneys, with extensive hemorrhages throughout, were noteworthy. Microscopically, this was verified, practically every glomerulus being obliterated. There were numerous hemorrhages in the lung and around the central vein of the liver. The bone marrow was hyperplastic. This hyperplasia included mainly the leucocytic series. There were no evidences of dissolution of the vascular bed of the bone marrow.

The outstanding features in Rabbit H-104 were (1) the prompt response to the injections, evidenced by the increase in reticulated cells and nucleated red blood cells and the rise in the number of erythrocytes and hemoglobin, and (2) the final hemorrhages. Possibly the kidneys were damaged before injections were begun, but there seems to to be no doubt that the last injection was the cause of the extensive hemorrhages.

The striking similarity in the picture of blood regeneration after hemorrhage and the appearance of normoblasts in the peripheral blood after injection of gum shellac solution is well illustrated in No. H-104 and healthy companion animals. In No. H-104 a few normoblasts were present in the blood 3 hours after the first injection, while in healthy animals they did not appear till after 24 hours. After hemorrhage in rabbits nucleated red blood cells appear in 24 to 48 hours (25–27). Usually the normoblastic crisis after hemorrhage is of short duration, while after gum shellac injection the presence of nucleated red blood cells in the peripheral blood stream was more prolonged.

A healthy rabbit treated in the same way as No. H-104 did not show any signs of illness after the fourth dose. To see whether hemorrhage could be produced, 4 cc. of a 2.2 per cent solution (double previous doses) was given 6 days after the fourth dose. When killed 24 hours later a few small hemorrhages were found in the lungs and in the bone marrow. The latter had been suspected from the appearance of the peripheral blood. The white blood cells had risen from 14,000 to 98,000 cells per c.mm. and many immature forms were present.

As hemorrhages had not been a feature of the previous experiments in which the injections had been given every 2nd day, they seemed to be the result of injections after long intervals apparently after the animal had been sensitized to the drug. To test this theory, one animal was injected in the same manner as No. H-84 (see Chart 2). 40 days after the administration of the last consecutive dose the equivalent of a single previous dose was given. When killed the following day, considerable hemorrhages were found in the bone marrow, corroborated before death by the appearance of immature cells of both the

leucocyte and erythrocyte series in the peripheral blood. Rabbit H-102 was given six doses of 4 cc. each of the 1.1 solution. 16 days later another dose produced no ill effect. Hemorrhages in the bone marrow were found, however, after a similar dose administered 14 days later. In this animal the surface of the kidney was studded with what appeared to be hemorrhagic spots. The microscopical examination revealed this to be an extreme dilatation of the blood vessels in about 50 per cent of the glomeruli, amounting to dissolution of the vascular bed in a few instances. The bladder urine showed blood microscopically.
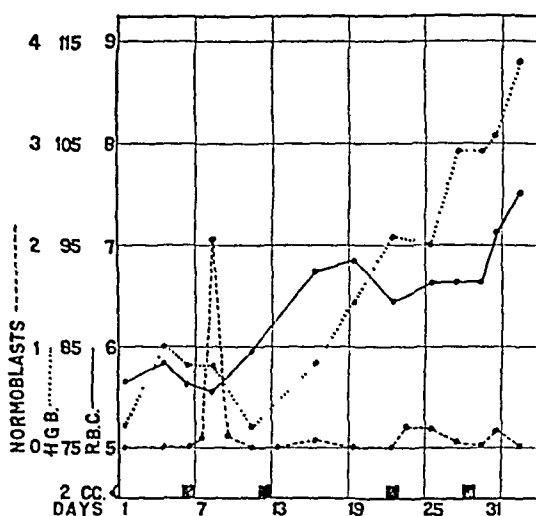
CHART 5. Rabbit H-105. Healthy animal given 2 cc. doses of 1.1 per cent gum shellac solution at long intervals. Normoblastosis present, but slight. No hemorrhages produced.

It is to be noted that in all these animals showing hemorrhages, the red blood cells and the hemoglobin had risen above the initial level. The fact remains, however, that the administration of 4 cc. doses of the 1.1 per cent solution at long intervals was toxic to the endothelium of the sinuses of the bone marrow and the vessels of the glomeruli after a certain time interval. With the same dosage as above (4 cc. of 1.1 per cent solution) given in divided doses at hourly intervals once a week, no ill effects were observed.

The effect of smaller dosage of the 1.1 per cent solution is illustrated in Chart 5, Rabbit H-105.

Four 2 cc. doses were given over a period of 26 days. At the end of the experiment the animal was in excellent condition. The bone marrow was moderately hyperplastic in structure. Leucocytic hyperplasia predominated, although at the edges of the cross-section there was considerable erythrocytic hyperplasia, the cells being mainly in the normoblastic stage. Blood vessels were intact, wide open, lined with a swollen endothelium wherever endothelium could be seen, otherwise with nucleated red blood cells in all stages of development.

In conjunction with Mr. Ralph Wheeler the effect of a certain quantity of gum shellac given in a definite time was obtained. As far as possible the conditions were kept constant, the variables being the time interval and the size of the dose. Medium sized doses given every 2nd day were found to be more effective than larger doses twice a week or smaller doses every day.

DISCUSSION.

From the findings described above, it is evident that gum shellac is an agent which markedly influences the hematopoietic organs. In common with many drugs this substance was fatally toxic in strong doses, and without any demonstrable effect in minimal doses. Medium strength solutions produced normoblastosis, accompanied by a period of slight decrease of red blood cells and hemoglobin, concomitant with the greatest outpouring of nucleated red blood cells. This normoblastosis was either preceded or followed by a rise in erythrocytes and hemoglobin above the initial levels which occurred during the period of injections or as soon as the injections were discontinued. Microscopically, an erythrocytic hyperplasia of the bone marrow was found immediately after discontinuation of the injections, and there was a return to normal conditions after variable intervals of time. With small doses the nucleated red blood cells in the peripheral blood stream were numerically less; the decline in erythrocytes and hemoglobin was minimal or altogether absent; the rise of erythrocytes and hemoglobin during and after the administration was, in many instances, greater than with medium sized doses. Hemorrhages,

mainly in the bone marrow and occasionally in the kidney glomeruli, were obtained under two sets of circumstances: (1) With a fatal dose in one animal which prior to injection apparently had a hyperplastic bone marrow. (2) *a*, After the fourth or fifth medium sized dose administered at intervals of from 4 to 19 days, and *b*, after an injection following a rest period preceded by a course of continuous injections. The latter phenomena indicated some kind of sensitization of the vascular endothelium. A precipitin reaction with gum shellac was obtained both with normal serum and serum from animals sensitized with gum shellac solution.

The explanation of the stimulation produced must be sought either in an increased blood destruction and compensatory regeneration, or in a direct stimulation of the bone marrow.

The concept suggested by Morawitz (28) that products of red blood cell destruction may normally stimulate the bone marrow is an attractive one, but it does not explain blood regeneration after hemorrhage. Some authors (7, 11) have suggested that splenic extract increases the blood destruction, a hormone being formed from these broken down cells capable of stimulating the bone marrow. Small doses of hemolytic serum subcutaneously (29), arsenic (30), and toluylenediamine (31), both hemolytic toxins, produce an increase of erythrocytes. Blood regeneration after destruction of red blood cells by phenylhydrazin is more rapid than after hemorrhage (32).

The evidences of blood destruction after injection of gum shellac are meager. Animals examined at frequent intervals for the first 24 hours after injection showed no decline but rather a rise in erythrocytes and hemoglobin. After several injections there was a decline in red blood cells and hemoglobin, concomitant with the presence of a great number of nucleated red blood cells in the peripheral blood, but this decline did not amount to an actual anemia which would explain the marked increase in the production of erythrocytes. The smears showed no undue increase of fragmentation. With the method used no appreciable increase of iron was found in the tissues. Hemoglobinuria was not observed. The concept of an appreciably increased blood destruction after injections of gum shellac solution can, therefore, be dismissed at least tentatively.

As evidences of stimulation of the bone marrow have been primarily

sought in the peripheral blood without examination of the bone marrow the facts reported in the literature are difficult to evaluate. Excluding anemia, erythrocytic hyperplasia has been produced by low oxygen tension (18, 19), and various colloids, such as "elektroferrol" (17) and collargol (4, a, 33). In this connection it may be of interest to note that Metchnikoff in 1900 (34) observed the relation between increased phagocytosis and new formation of red blood cells in lepers injected with horse serum.

The evidences for direct stimulation of the bone marrow in the present investigation are suggestive although not conclusive. The substance injected was taken up, at least partly, by the phagocytic cells in the hematopoietic organs. Whether this substance, visible in the phagocytic cell, is the actual stimulant of red blood cell formation or an accessory cannot be determined from data in hand. That the endothelial cells are strongly affected is evidenced by their hypertrophy and the hemorrhages, produced by a fatal dose in one animal with a hyperplastic marrow and after the administration of the drug at long intervals in others. It is of interest to note that the hemorrhages produced occurred in all instances after erythropoietic stimulation. The response varied with the state of activity of the endothelial cells; i.e., an inactive cell was stimulated, while a hyperactive cell responded to the same dosage with overstimulation which brought about complete permeability or cell death.

Gum shellac seems to be a substance which stimulates the blood-forming organs to a higher degree than any other substance heretofore reported. Its complexity, toxicity, and the possible necessity for intravenous administration renders therapeutic application as yet undesirable. The effect of this drug on the endothelium may throw some light on the various reactions of the hematopoietic system.

CONCLUSIONS.

1. The effect produced by intravenous administration of gum shellac solution varied directly with the strength of the solution and the amount injected.

(a) Strong solutions were intensely toxic and fatal to all the animals injected. Toxicity was abolished by the presence of carbon particles in the solution.

(b) Medium strength solutions produced a marked normoblastosis followed by an erythroblastosis if injections were continued, accompanied either by an initial rise of red blood cells and hemoglobin followed by a decrease or an initial decline followed by an increase. The decrease of erythrocytes and hemoglobin did not amount to an actual anemia, and was usually concomitant with the greatest outpouring of nucleated red cells.

(c) Small doses elicited the same kind of response except that the normoblastosis was less marked, the decrease of red cells and hemoglobin minimal or absent, the increase of red blood cells and hemoglobin marked in most instances.

(d) Minimal doses elicited no appreciable reaction.

2. The bone marrow in animals killed after a course of injections showed intense erythrocytic hyperplasia. This disappeared after a variable length of time with a return of the bone marrow to apparently normal condition.

3. Toxic effects with non-fatal doses in form of hemorrhages were produced mainly in the bone marrow and occasionally in the kidney under the following conditions.

(a) After one injection in an animal with hyperplastic marrow (hemorrhages in the bone marrow only).

(b) After the fourth and fifth dose when administered at long intervals (4 to 19 day intervals).

(c) After one injection given 40 days after several successive injections (one animal only).

4. Small and divided doses administered at long intervals produced no apparent ill effect.

5. Subcutaneous administrations did not elicit any marked systemic reactions. Locally there was induration and edema.

6. The conclusion was drawn that gum shellac solution stimulates markedly the production of erythrocytes in the bone marrow. The mechanism of stimulation is not clear, but it seems unlikely that it is due to destruction of red blood cells in the peripheral blood stream.

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EXPLANATION OF PLATE 26.

FIG. 1. Rabbit H-83. General appearance of bone marrow immediately after discontinuation of injections of gum shellac solution showing marked erythrocytic hyperplasia.   × about 685.

FIG. 2. Rabbit H-114.   Shows a blood vessel lined with nucleated red cells in various stages of maturation after a short course of injections of 1.1 per cent gum shellac solution.   × about 820.
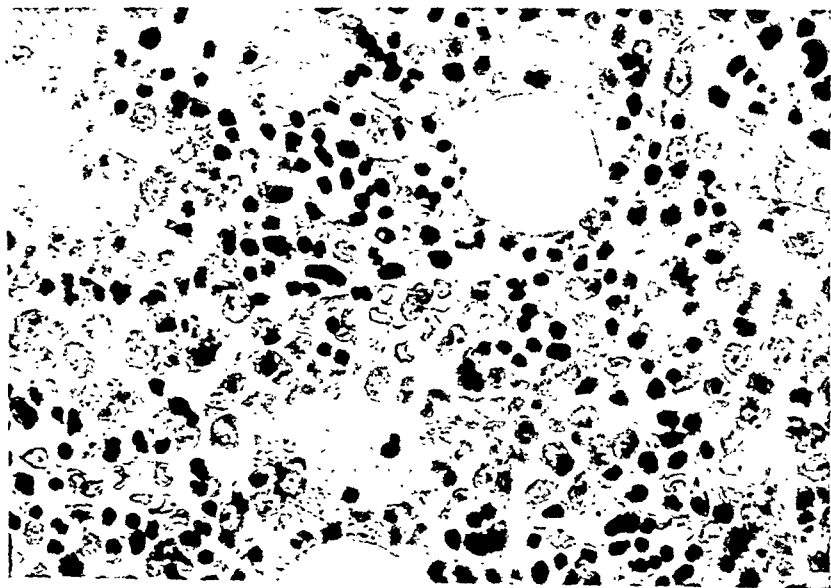
FIG. 1.
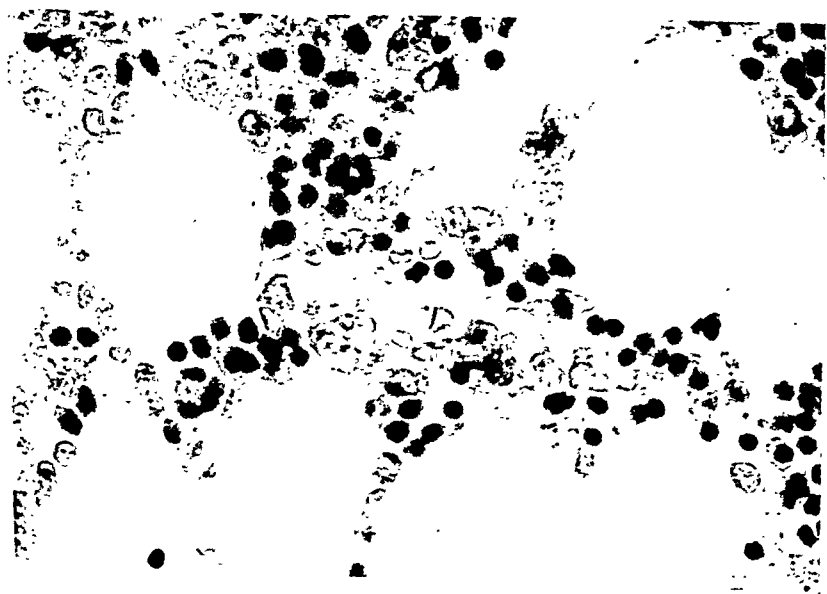
FIG. 2.

(Muller: Experimental bone marrow reactions. III.)

THE EFFECT OF GUM SHELLAC SOLUTION ON THE SURFACE TENSION OF RABBIT SERUM.

By J. M. HAYMAN, Jr.*

(*From the Thorndike Memorial Laboratory, Boston City Hospital, Boston.*)

One of the interesting points in the action of the gum shellac solution studied by Muller (1) is the short interval after a single intravenous injection in a rabbit at which changes in the peripheral blood picture may be detected. The reaction rapidly subsides, and within a few days the blood picture has returned to normal.

It seemed possible that one factor in this series of events might be the changes in the physicochemical equilibrium of the plasma produced by the gum shellac solution which facilitated the removal of cells from the marrow sinuses. Among such changes, any which resulted in alterations of the properties of cell-serum interface might be most important.

The existence of such changes has not been determined directly. An indication of changes in the physicochemical state of the plasma after injection of this gum in rabbits was sought in measurements of the surface tension of the serum.

Du Noüy (2) has shown that the most satisfactory method of studying the surface tension of serum consists in measurements by the du Noüy tensiometer of the time-drop in diluted sera; that is, the difference between the initial surface tension of the diluted serum and that after equilibrium is reached. Since blood serum contains substances which lower surface tension, when pure serum is exposed to air the surface energy of its free surface decreases progressively for about 20 minutes. When serum is diluted with physiological salt solution the decrease of surface energy is less rapid, equilibrium being reached in approximately 2 hours. In a study of the changes in the surface

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TABLE I.

Surface Tension of Dilutions of Rabbit Serum after Intravenous Injection of Gum Shellac Solution.

Rabbit No.	Dilution of serum	Before injection			After injection of gum											
					½ hour			24 hours			48 hours			144 hours		
		Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop
		dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes
7	1: 5,000	74.4	65.3	9.1	74.3	60.5	13.8									
	1:10,000	75.5	67.6	7.9	75.1	61.4	13.7									
	1:15,000	75.5	71.2	4.3	75 5	64.3	11.2									
9	1: 5,000	72.8	64.5	8 3	75 4	61.1	14.3				75.4	64.7	10.7			
	1:10,000	73.8	65.5	8 3	75.6	63 4	12.2				75.6	66.2	9.4			
	1:15,000	74.5	68.2	6 3	75.8	64.6	11.2				75.8	68.8	7.0			
12	1: 5,000	70.2	64.8	5 4				75.5	62.5	13.0						
	1:10,000	72.8	66.2	6.6				75 3	64.6	10.7						
	1:15,000	74.3	67.6	6.7				75.5	65.4	10.1						
3	1: 5,000	72.3	63.2	9.1				76.4	64.5	11.9	76.2	61.2	15.0	75.3	76.0	8.3
	1:10,000	76.2	65.7	10 5				76.5	67.0	9.5	76.5	62.5	14.0	76.6	67.6	9.0
	1:15,000	76.2	68.1	8.1				76.5	65.2	11.3	76.4	65.7	10.7	76.6	70.6	6.0
5	1: 5,000	75.3	67.0	8.3							75.1	60.2	14.9	72.8	64.5	8.3
	1:10,000	76.6	67.6	9 0							76.2	61.1	15.1	73.8	65.5	8.3
	1:15,000	76.6	70.6	6.0							75.4	60.5	14.9	74.5	68.2	6.3

11	1: 5,000	73.3	64.1	9.2					72.0	56.0	16.0	75.6	66.4	9.2
	1:10,000	75.1	65.4	9.7					76.4	61.0	15.4	75.8	67.0	8.8
	1:15,000	75.3	66.2	9.1					76.1	63.4	12.7	75.8	68.0	7.8
15	1: 5,000	73.0	65.5	7.5					76.0	60.2	15.8			
	1:10,000	74.4	68.7	5.7					76.2	60.4	15.8			
	1:15,000	75.2	68.6	6.6					76.3	64.3	12.0			
16	1: 5,000	73.4	62.5	10.6										
	1:10,000	74.5	65.7	8.8										
	1:15,000	74.3	66.8	7.5										

tension of rabbit serum which accompany immunization, du Noüy (3) adopted a technique which involved the dilution of the serum to 1:10,000, a volume of 2 cc. of liquid, watch-glasses giving a diameter of about 4 cm. of free liquid surface with this volume of fluid, and measurement of the decrease in surface tension, or time-drop, in 2 hours. Surface tension was measured by the du Noüy tensiometer.

Methods.

We have followed du Noüy's technique. Great care was taken to have all glass with which serum, its dilutions, or distilled water came in contact strictly clean. Glassware was cleaned by boiling in sulfuric acid—dichromate mixture. Watch-glasses were usually washed and used on the same day; occasionally watch-glasses were used which had been washed the preceding day. All water was glass-distilled. Salt solution (0.9 per cent) used for diluting serum was made from Kahlbaum's NaCl, and when evaporated in a clean watch-glass gave crystals similar to those pictured by du Noüy (4). The surface tension of the salt solution was repeatedly checked, and did not show any fall in 2 hours.

Only rabbits having a normal time-drop were used (3). Blood was obtained by cardiac puncture. 5 cc. of a 1.1 per cent gum shellac solution were injected into an ear vein, and blood obtained after intervals of 30 minutes, 24, 48, and 144 hours. We are indebted to Dr. Muller for a supply of the gum shellac solution which she used. In most experiments a second rabbit was bled at the same time and at the same intervals as the experimental animal. This control served the double purpose of showing the effect of repeated bleedings of 3 cc. on the surface tension of the serum, and of showing that observed changes in the time-drop were not due to chance variations in room temperature, in the particular batch of salt solution used, or to other technical errors.

Three dilutions of serum, 1:5,000, 1:10,000, and 1:15,000 were made from the serum obtained after centrifugation. The serum was diluted 1:100 in volumetric flasks, and further dilutions made in test-tubes. Pipettes were not recalibrated. These three dilutions, rather close together, were chosen not only as a check on the accuracy of the technical manipulations, but because it seemed possible that if the gum shellac solution had any demonstrable effect on the surface tension of serum, it might be manifested by a change in the concentration at which the greatest time-drop occurred. With the technique used du Noüy has found that the maximum time-drop with normal serum takes place around 1:10,000 dilution.

The watch-glasses were brought under the tensiometer ring by means of the turntable described by du Noüy. For the use of this apparatus and for watch-glasses of approximately the proper curvature, I am indebted to the great kindness of Dr. du Noüy's laboratory. Care was taken to increase the torsion on the tensiometer wire uniformly and slowly. The room temperature was maintained as close to 23°C. as possible.

TABLE II.

Surface Tension of Dilutions of Rabbit Serum after Repeated Bleeding.

Rabbit No.	Dilution of serum	First bleeding			Subsequent bleedings after ¼ hour			24 hours			48 hours			144 hours		
		Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop
		dynes	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*
8	1: 5,000	74.6	66.5	8.1	73.3	64.1	9.2									
	1:10,000	75.4	68.5	6.9	75.1	65.4	9.7									
	1:15,000	75.5	70.3	5.2	75.3	66.2	9.1									
10	1: 5,000	71.4	63.6	7.8	74.0	63.2	10.8									
	1:10,000	73.0	65.7	7.3	75.4	65.6	9.8									
	1:15,000	74.1	67.1	7.0	75.7	66.3	8.4									
4	1: 5,000	74.3	63.9	10.4				73.3	63.7	9.6	73.7	63.9	9.8			
	1:10,000	76.3	66.5	9.8				76.2	66.5	9.7	75.2	67.9	7.3			
	1:15,000	76.4	68.2	8.2				76.6	68.7	7.9	75.6	68.9	6.7			
6	1: 5,000	75.6	66.8	8.8							70.9	60.7	10.2	75.6	66.8	8.8
	1:10,000	76.5	68.8	7.7							71.5	63.2	8.3	76.5	68.8	7.7
	1:15,000	76.7	71.4	5.3							71.6	65.1	6.5	76.7	71.4	5.3
13	1: 5,000	68.2	61.0	7.2	73.9	64.4	9.5				71.6	63.5	8.1	71.4	63.6	7.8
	1:10,000	68.8	60.8	8.0	75.4	67.1	8.3				74.4	65.4	9.0	73.0	65.7	7.3
	1:15,000	71.5	65.2	6.3	75.5	68.5	7.0				74.1	64.8	9.3	74.1	67.1	7.0
14	1: 5,000	74.3	63.3	11.0	71.7	62.6	9.1									
	1:10,000	75.2	66.4	8.8	72.5	63.6	8.9									
	1:15,000	75.5	67.8	7.7	74.6	64.6	10.0									

J. M. HAYMAN, JR.

EXPERIMENTAL DATA.

The estimations of the surface tension of diluted serum before and after the injection of gum shellac solution in eight rabbits are presented in Table I.   The figures given are the average of two measurements at 1:5,000 and 1:15,000 dilutions and of three at 1:10,000.   In two animals blood was drawn $\frac{1}{2}$ hour after injection of gum; in four animals, 24 hours; in four, 48 hours; and in two, 144 hours after injection of gum.
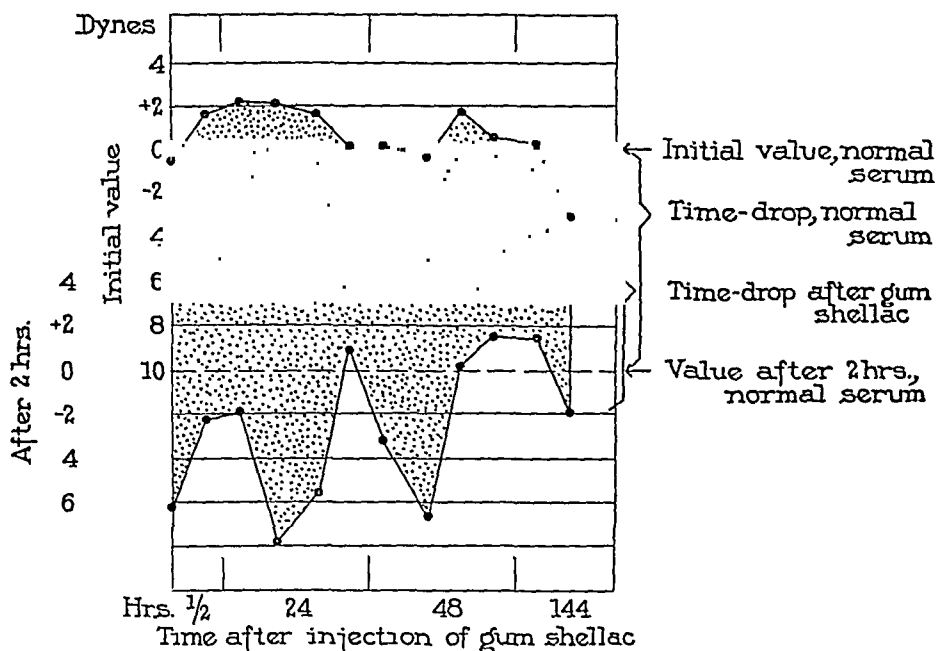
TEXT-FIG. 1. The stippled area represents the time-drop of 1:10,000 dilution of serum after the injection of gum shellac while the area between the two parallel lines corresponds to the time-drop of normal serum.

There was a distinct increase in the value of the time-drop $\frac{1}{2}$ hour after injection in both animals; an increase in three of four 24 hours after injection; and in two, 48 hours after injection.   In both animals bled after 6 days, the time-drop had returned to normal.

The data of six control experiments are presented in Table II. In order to determine whether the injection of any colloid was followed by change in the surface tension of serum, one animal was given 5 cc. of 6 per cent gum acacia solution and one, 5 cc. of diphtheria

antitoxin intravenously after the first bleeding.   No change in surface tension of the serum was detected.

<div align="center">TABLE III.</div>

Change in Time-Drop of 1:10,000 Dilution of Serum after Intravenous Injection of Gum Shellac Solution.

Rabbit No.	Before injection	After injection of 5 cc. of gum shellac solution			
		½ hour	24 hours	48 hours	144 hours
	dynes	*dynes*	*dynes*	*dynes*	*dynes*
7	7.9	13.7			
9	8.3	12.2		9.4	
12	6.6		10.7		
3	10.5		9.5	14.0	9.0
5	9.0			15.1	8.3
11	9.7			8.8	
15	5.7		15.4		
16	8.8		15.8		
Average......	8.3	12.9	12.8	11.8	8.6

Change in Time-Drop of 1:10,000 Dilution of Serum with Repeated Bleedings and Injection of Other Colloids.

Rabbit No.	First bleeding	Subsequent bleedings after			
		½ hour	24 hours	48 hours	144 hours
	dynes	*dynes*	*dynes*	*dynes*	*dynes*
8	6.9	9.7			
10	7.3	9.8		7.3	
4	9.8		9.7	8.3	7.7
6	7.7			9.0	7.3
13	8.0*		8.3		
14	8.8**		8.9		
Average......	8.0	9.7	8.9	8.2	7.5

* 5 cc. of 5 per cent acacia intravenously immediately after bleeding.
** 5 cc. of diphtheria antitoxin immediately after bleeding.

Inspection of these tables shows that the maximum time-drop was recorded at 1:5,000 more frequently than at 1:10,000. This we believe to be due to technical errors. The curvature of the watch-

glasses, although approximately uniform, was not calibrated, so that a monomolecular layer at the surface may not have been possible at 1:10,000 in some instances.

The changes in time-drop at 1:10,000 dilution in both control animals and after the injection of gum shellac solution are summarized

TABLE IV.

Rise of Surface Tension in Function of Time after Drop Due to Addition of 0.5 cc. Gum Shellac Solution.

Time	Surface tension			
	Concentration of serum			
	Pure	1:10	1:20	1:100
	dynes	*dynes*	*dynes*	*dynes*
Before adding gum............	59.0	61.7	60.7	60.8
After " "	49.6	50.8	50.8	50.6
" 2 min.................	54.0	55.1	53.5	51.9
" 5 "	56.8	57.0	56.7	54.4(?)
" 10 "	56.6	56.3	57.7	
" 30 "	55.3	55.0	57.0	51.6

Rise of Surface Tension in Function of Time after Drop Due to Addition of 0.025 cc. Gum Shellac Solution.

Time	Surface tension		
	Concentration of serum		
	Pure	1:10	1:20
	dynes	*dynes*	*dynes*
Before adding gum.....................	60.8	60.3	60.1
After " "	53.6	53.5	50.3
" 2 min.........................	56.0	57.7	55.0
" 5 "	56.4		56.2
" 30 "	56.4	58.0	58.4

in Table III and Text-fig. 1. In this figure the stippled area represents the time-drop of 1:10,000 dilution of serum after injection of gum, while the area between the two parallel lines corresponds to the time-drop of normal serum. In constructing the figure (3), the initial value of serum before injection of gum was taken as 0, thus representing the axis of abscissæ, and difference between the initial values

of surface tension of serum of the same animal before and after injection of gum shellac solution were plotted with respect to this axis. The same procedure was followed in plotting final values. When these two sets of figures are plotted on one graph, however arbitrary the distance may be between the two parallel abscissæ representing the initial value of normal serum and the value after 2 hours, the ratio between this area and that included between the curves representing initial and final values of serum after injection of gum, expresses the ratio between the values of the time-drop in both cases.
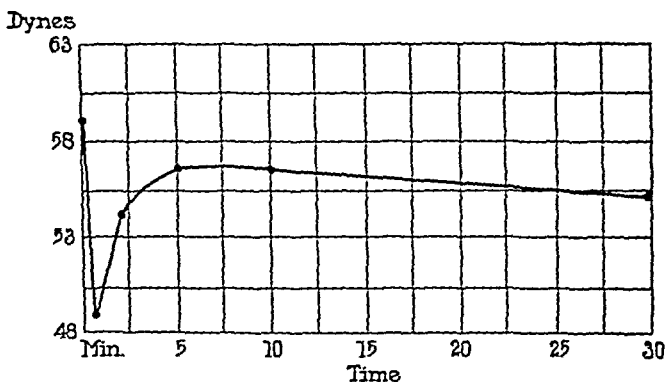
Text-Fig. 2. Rise of surface tension of pure serum after drop due to addition of 0.05 cc. of gum shellac solution.

Recovery after Lowering of Surface Tension by Gum Shellac Solution.

Du Noüy (5) has shown that after the addition of minute amounts of sodium oleate to serum there is a rapid recovery of surface tension due to the adsorption of the oleate by the serum molecules or micellæ. After the addition of 0.05 cc. of gum shellac solution to 2 cc. of pure or diluted serum there was rapid fall of about 10 dynes in surface tension and prompt recovery in dilutions less than 1:100 (Table IV and Text-fig. 2). The initial fall is only about half that produced by minute amounts of sodium oleate. The addition of 0.025 cc. of gum shellac solution produced a distinct, but less marked fall in the surface tension. The surface tension of the gum shellac solution itself was 46.8 dynes. The addition of 0.05 cc. of this solution to 2 cc. of 0.9 per cent NaCl lowered the surface tension from 76.2 to 50.1 dynes: 1 hour later it was 49.9 dynes.

This experiment shows that the gum acts like a surface-active substance. Since the du Noüy phenomenon of recovery is observed, the gum molecules must be adsorbed by the serum molecules.

A crude attempt was made to detect adsorption of surface-active substances from the gum shellac solution on red blood cells by their rate of sedimentation. No differences could be detected between the rate in blood to which 3 per cent of gum shellac solution was added, or in blood drawn 30 minutes after intravenous injection of gum shellac solution, and in the controls.

CONCLUSIONS.

Following the intravenous injection of a gum shellac solution which alters the peripheral blood picture there is an increase in the time-drop of the diluted serum. This is believed to indicate changes in the physicochemical state of the plasma.

This gum shellac solution behaves as a surface-active substance; its effect on the surface tension of serum is hindered by the du Noüy phenomenon of adsorption on serum molecules.

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ETIOLOGY OF OROYA FEVER.

VIII. Experiments on Cross-Immunity between Oroya Fever and Verruga Peruana.

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A common etiologic origin of Oroya fever and verruga peruana was first indicated experimentally by the production of verrucous lesions in monkeys (*Macacus rhesus*) by inoculation of cultures of *Bartonella bacilliformis* isolated from the blood in a fatal case of Oroya fever.[1] Subsequent experiments with the same strain[2,3] gave evidence that the type of disease induced by the parasite was dependent upon two factors, the virulence of the strain, and the degree of susceptibility of the individual animal, various grades of infection being induced in the experimental animals, from a moderate or extensive local skin affection to a severe systemic disease terminating in fatal anemia. More recently,[4] there has been isolated from the skin lesion of verruga peruana a microorganism morphologically and serologically identical with *Bartonella bacilliformis* and having similar pathogenic properties. It remained to be proven that animals which had recovered from infection with the Oroya strain of *Bartonella bacilliformis* would be able to resist infection with the verruga strain.

The disease induced by *Bartonella bacilliformis* in *M. rhesus* is chronic in nature, but complete recovery may take place and is evident from (1) the subsidence of the febrile reaction, (2) the disappearance of the skin lesions, (3) the sterility of the blood and lymphatic glands, as shown by cultivation experiments, and finally (4) immunity to the homologous strain. All of the ten monkeys

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

[4] Noguchi H., *J. Exp. Med.*, 1927, xlv, 175.

(nine *Macacus rhesus* and one chimpanzee) selected for the present investigation had been shown by cultivation of blood and lymphatic gland tissue to be free from organisms, and five of them had been proved immune to the homologous (Oroya) strain by one or more previous immunity tests. The final test of immunity against both strains was made with suspensions of nodular tissue from *M. rhesus* 41 (verruga strain, second passage) and *M. rhesus* 36 (Oroya strain, fifth passage), the Oroya strain being inoculated on the right eyebrow and abdominal skin, intradermally and by scarification, the verruga strain similarly on the left side. Both suspensions induced well developed lesions in a control monkey, the verruga strain being decidedly the more virulent locally, as shown in the protocol of this animal. In the ten animals which had previously passed through a course of infection with the Oroya strain no signs of infection could be induced.

M. rhesus 1 had been inoculated on Nov. 16, 1925, intradermally and intravenously, with the first generation cultures of the blood of Case S. A. 15.[1] *Bartonella bacilliformis* was cultivated from the blood several times during the course of disease, and nodules arose at the site of intradermal inoculation. The lesions had disappeared within 2 months, and on Feb. 22, 1926, cultures of blood and lymphatic gland tissue were negative. Mar. 9, 1926, the animal received intravenously and intradermally a mixture of cultures and a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 31, and Apr. 13 proved sterile. On Apr. 29 the animal was inoculated again, intradermally and by scarification, with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] On May 19, minute lesions appeared to be developing on the eyebrows, but the slight indurations disappeared, and blood and lymphatic gland tissue taken on June 1 yielded no cultures. The final test with both Oroya and verruga strains was made on June 3, the inoculation being made intradermally and by scarification. No lesions developed.

M. rhesus 2 was first inoculated Dec. 8, 1925, and had passed through a protracted course of infection with the Oroya strain.[1] The blood had yielded cultures on several occasions, and the nodules at the sites of intradermal injection on the eyebrows were still present on Mar. 27. They had disappeared by Apr. 27, and the blood and lymphatic gland tissue taken on that date were negative by culture test. On Apr. 29 the animal was reinoculated with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] No signs of local or systemic infection appeared, and blood and lymphatic gland tissue proved sterile on June 1. On June 3 the animal was inoculated intradermally and by scarification with both the Oroya and the verruga strains. Blood and lymph node cultures made June 22 and July 13 proved sterile. No local lesions developed.

M. rhesus 3 had been inoculated with the same material as *M. rhesus* 2 and had passed through a similar course of disease. Only traces of the local lesions were visible on Mar. 8, 1926. On Mar. 9 the animal was inoculated intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 22 yielded cultures of *Bartonella bacilliformis*, and small but definite lesions developed at the sites of local inoculation. These had disappeared by Apr. 28, however, and the blood was sterile. On Apr. 29 a suspension of nodular tissue from *M. rhesus* 25[2] and cultures were introduced intradermally and by scarification. No local reactions resulted, and on May 19 and June 1 blood and lymphatic gland tissue were sterile. On June 3 the animal received the inoculation of both the Oroya and the verruga strains, intradermally and by scarification. The blood remained sterile, and no local lesions developed.

M. rhesus 12[5] had been inoculated on Jan. 19, 1926, intravenously, subcutaneously, and by scarification with cultures derived from Monkeys 4, 6, and 7. The blood was positive by culture on two occasions, and there was high fever for 1 week, but the local lesions were never large, and they disappeared within a month. On Mar. 25 blood and lymphatic gland tissue were sterile. On Apr. 22 the animal was inoculated with a suspension of nodular tissue from *M. rhesus* 18,[2] cultures derived from Monkeys 7 and 20, and citrated blood of *M. rhesus* 25.[2] The blood was negative by culture on May 27, and lymphatic gland tissue was negative on June 1. No lesions developed. On June 3 the final test with both the Oroya and the verruga strains was made. The blood and lymphatic tissue were sterile when tested on June 22, and no lesions developed.

M. rhesus 16 had received two injections (Feb. 10 and Feb. 15, 1926) of killed cultures of *Bartonella bacilliformis*. No reaction resulted. On Mar. 6 active suspensions of the skin lesions from *M. rhesus* 5[2] and *M. rhesus* 18 were injected intradermally and by scarification. By Apr. 5 a large nodule had developed on the abdomen. On Apr. 14 it was larger and deep cherry red. By Apr. 28 the lesion had practically healed. On Apr. 29 the animal received cultures and a suspension of nodular tissue of *M. rhesus* 25[2] intradermally. Small papules appeared at the sites of injection on May 22, but they disappeared, and on June 1 no lesions were apparent. Blood and lymphatic gland tissue taken on this date were sterile. The final test with both the Oroya and the verruga strains was made on June 3. No lesions developed. The blood was sterile when tested on June 21.

M. rhesus 18[2] had been inoculated on Feb. 15, 1926, intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 5. Extraordinarily extensive lesions had developed by Mar. 17, and blood taken on Mar. 18 yielded cultures of *Bartonella bacilliformis* in dilutions as high as 1:100,000. The lesions persisted for many weeks but had become very small, pale, and fibrous by the middle of June. Blood and lymphatic gland tissue taken on May 15 yielded no cultures. No reaction followed the inoculation of the Oroya and verruga strains on June 3, and on June 22 the blood and lymphatic tissue were sterile by culture test.

M. rhesus 22, inoculated Mar. 5, 1926, intradermally and by scarification on

[5] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

eyebrows and abdomen with nodular tissue of *M. rhesus* 5.   Apr. 26, blood diluted 1:1,000 yielded cultures of *Bartonella bacilliformis*.   A nodule had developed on the right eyebrow, and by May 6 it was very large and pedunculated.   The lesion had disappeared by June 1, and blood and lymphatic gland tissue taken on that date were sterile by culture test.   The final test with the Oroya and verruga strains was made on June 3.   No lesions developed during 4 weeks of observation, and the blood was sterile by culture test on June 22.

M. rhesus 26[5] had been infected by the bites of four ticks previously fed on *M. rhesus* 18, the ticks having been transferred from Monkey 18 to Monkey 26 on Mar. 29, 1926.   Lymphatic gland tissue (inguinal) taken on Apr. 14 yielded cultures of *Bartonella bacilliformis*, as did also blood taken on Apr. 24.   On May 28 blood and lymphatic tissue were both sterile.   The animal was inoculated on June 3 with both the Oroya and the verruga strains.   No lesions developed during 6 weeks of observation.   The blood was negative by culture test on June 22.

M. rhesus 31 was inoculated intradermally Apr. 5, 1926, with a suspension of testicular tissue of Rabbit 1744[6] and nodular tissue of Dog 1.[6]   Blood taken on Apr. 28 yielded cultures in a dilution as high as 1:10,000.   The nodules were well developed at this time.   May 22, the nodules were reduced in size.   May 28, blood culture sterile.   June 1, lesions had disappeared, blood and lymphatic tissue failed to yield cultures.   June 3, final test with Oroya and verruga strains.   No reaction. Blood sterile on June 22.

Chimpanzee (*Pan leucoprymnus*), had been inoculated Jan. 29, 1926, intradermally, subcutaneously, and by scarification, with mixture of cultures and suspension of nodule of *M. rhesus* 3.   Blood positive in dilution of 1:10,000 Feb. 23, 1:100 Mar. 12.   Large nodules developed on eyebrows and abdomen.   The animal had recovered completely by May 1, and neither blood nor lymphatic gland yielded cultures of *Bartonella bacilliformis*.   On June 3 the animal was tested for immunity against the Oroya and verruga strains in the same way as the nine *rhesus* monkeys. No lesions developed, and blood culture was negative on June 28.

The following protocol illustrates the effects produced in a non-immune control by inoculation of the materials used on June 3 for the final test against the Oroya and verruga strains of *Bartonella bacilliformis*.

M. rhesus 45 was inoculated June 3, 1926, with the same materials and in the same manner as the recovered monkeys, the Oroya strain being inoculated on the right eyebrow and abdominal skin, the verruga strain at corresponding sites on the left side.   Several minute nodules were recognizable at the sites of inoculation on

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[6] Both the testicular tissue of the rabbit and the nodule of the dog yielded cultures of *Bartonella bacilliformis;* the report of these and other experiments will be published later.

the left side within 10 days and had attained considerable size (8 × 20 mm.) 32 days after the inoculation. The reactions at the sites of inoculation with the Oroya strain did not become noticeable until 3 weeks after inoculation. The nodules reached their maximum size (4 × 8 mm.) by the middle of July, never attaining the extent of the lesions on the left side. All the lesions were similar in character, however, being of the usual subcutaneous, *mular* type. Cultures made with blood withdrawn on June 28, 1926, 25 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. All the lesions had healed by the middle of August.

The experiments demonstrate that *rhesus* monkeys which have recovered from infection with the Oroya strain of *Bartonella bacilliformis* are completely immune to the verruga strain. The results warrant the conclusion that the strains from Oroya fever and verruga peruana are identical, and that both conditions are manifestations of infection with *Bartonella bacilliformis*.

The duration of the infection in the present series of animals varied from 2 to 5 months. The cultural test of lymphatic gland tissue appears to be extremely important in determining whether or not the animal is free from infection, as has already been shown in another series of experiments,[7] in which it was found that two animals of a group of four which had apparently recovered still harbored *Bartonella bacilliformis* in certain organs 48 and 58 days, respectively, after inoculation. In this earlier series of animals convalescence was established as early as 30 days in some instances, while in others the infection was still active 68 days after inoculation. The duration of a fatal infection varies from 25 to 57 days.[7]

SUMMARY.

Nine monkeys (*Macacus rhesus*) and a chimpanzee which had recently recovered from an infection with the Oroya strain of *Bartonella bacilliformis* were tested for immunity against the verruga strain of *Bartonella bacilliformis* as well as against the homologous strain. Complete immunity to both strains was demonstrated. The result establishes the identity of the strains and is in agreement with the result of comparative serological study.

[7] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

The criteria of recovery include not only the subsidence of febrile reactions and local lesions but also a negative result of cultural tests of blood and lymphatic gland tissue.

Recovery may occur as early as 1 month after inoculation, but in most instances a period of 2 to 5 months is required for the completion of convalescence.

A STUDY OF PNEUMOCOCCI ISOLATED FROM HORSES UNDERGOING PNEUMOCOCCUS IMMUNIZATION.

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In previous studies (1), the significance of tissue susceptibility to the pneumococcus was demonstrated experimentally in the development of the lesions of lobar pneumonia not only in very susceptible animals, but even in the highly immunized horse where extensive lesions of ulcerative infectious endocarditis were recorded. Many observations on the changes that occur in the pneumococcus growing in the presence of immune serum *in vitro* are found in the literature, and Rosenow (2, 3) early described the attenuation of pneumococci isolated from the lesions of human endocarditis. Endocardial lesions in horses undergoing immunization offer exceptional opportunities for this study.

In 1916, Stryker (4) studied the growth of pneumococci in their homologous immune sera, recording variation in agglutination, diminution in virulence, inhibition of capsule formation, increased susceptibility to phagocytosis, and changes in the absorptive power and antigenic properties of such cultures. Passage through susceptible animals restored these cultures to normal type. Griffith (5) also records the fact that virulent pneumococci are attenuated by growth in immune serum and that their immunological character is changed. He recognized alterations in colony development by distinguishing the virulent colony as "smooth" and the attenuated as "rough." Blake and Trask (6), also Reimann (7), have described similar changes in the pneumococcus grown in the presence of immune serum. Neufeld and Händel (8) observed that pneumococci were attenuated by cultivation at a temperature above 39°C. and by surface growth on unfavorable medium. More recently Yoshioka (9) has reported that pneumococci kept under conditions that lead to loss of virulence, such

as cultivation at 39°C., surface growth on unfavorable medium, and desiccation, suffer changes in morphology, cultural characteristics, capsule formation, lysis in bile, and especially in type specificity (agglutination).

It is the purpose of this paper to record observations made in the study of the pneumococci isolated from the blood stream in the course of pneumococcus immunization, or at autopsy on immunized animals which had died as a result of the development of pneumococcus processes in the heart or other organs. Such observations, presenting, as they do, definite evidence that there is an attenuation of the pneumococcus in the course of prolonged infectious processes in the tissues, are of special interest because pneumococcus infection, however serious and extensive it may be, is usually self-limited and terminates by crisis or rapid lysis and a complete resolution of the tissues. The opposite course, delayed resolution, prolonged balanced infection, and the persistence of the pneumococci, even in pure culture, for a long time, is occasionally found, although, in these conditions, secondary infections frequently develop.

Of all the pathogenic species, the pneumococcus is one of the most susceptible to changes in its environment. Examples of its sensitive reaction to environmental change are the rapidity with which its virulence is exalted by animal passage and, especially, the fluctuation in virulence which occurs in the course of 24 hours' growth in broth, a fluctuation directly related, apparently, to its vegetative activity (10). Although the alteration in the pneumococcus grown in the presence of immune serum, as recorded in the literature, appears to be due specifically to the action of the serum, it is difficult to determine to what extent it is effected by some specific interaction between the organism and its immune serum, and how much is brought about by the biologic changes which take place in a less favorable environment. Some of these changes are, possibly, of a specific nature, while others must be regarded as general in character.

Source of Cultures.

The cultures which are the subject of this study were isolated directly from horses undergoing immunization or from horses at

autopsy. The following descriptive notes concerning them have been abstracted from the laboratory records.

Strain 131.—Horse 131 was immunized by intravenous injection from September 26 to December 8, 1923, with a Type I culture, HL, obtained originally from Dr. G. W. McCoy of the Hygienic Laboratory, Washington, D. C. Following the last injection of 175 cc., the temperature of the animal was 104.4°F.; and 5 days later, 102.2°F. From a blood culture taken December 22, a Gram-positive, bile-soluble diplococcus which agglutinated in all three type sera was isolated. Later, another culture was obtained which gave similar reactions. January 6, 1924, the animal died. At autopsy, the lungs were found to be congested, with small areas of adhesion. In the heart, there was a marked thickening of one cusp of the mitral valve, ulceration and degeneration of the cusps of the aortic valve, with marked stenosis.

Strain 143.—Horse 143 was immunized with the same strain, I HL, from August 28, 1923, to July 17, 1924, when it was allowed to rest. Immunization was resumed October 29, 1924, with another and more virulent Type I strain, the standard Neufeld Culture 5. On December 24, 1924, 5 days after the last injection, the temperature was 101°F. and gradually rose to 104.6° on January 20, 1925. On January 2, a blood culture was taken from which was isolated a Gram-positive, bile-soluble diplococcus which grew like the pneumococcus in broth and fermented inulin, and which agglutinated non-specifically in all three type sera, as did the organism isolated from Horse 131. Other organisms which gave similar reactions were isolated from blood cultures 6 and 11 days later, but the growth of the last culture in broth was flocculent. The serum obtained from a bleeding of January 3 possessed a high protective titer when standardized for therapeutic use. January 14, the right hock was swollen and, on the 19th, valvular murmurs were noted. On the 20th, the temperature of the animal was 104.6°F., and, from a blood culture, another organism was obtained which was quite similar to the ones previously isolated and was also flocculent in its growth in broth. January 24, the animal died with ulcerative endocardial lesions. One cusp of the bicuspid valve was thickened; the other was thickened and covered with thick fibrinous exudate. The aortic valves showed marked ulceration and were covered with fibrinous exudate.

Strain 177-A.—The immunization of Horse 177 with the standard Type I No. 5 strain began July 7, 1924. November 4, the horse developed lameness. Its temperature ranged from 100–104.4°F. until it was bled out, January 30, 1925. January 24, a Gram-positive diplococcus with flocculent growth in broth was isolated from a blood culture. The agglutinative reaction of this organism was similar in character to that of the pneumococci isolated from the other horses. A mouse was inoculated with this culture, and the organism obtained at autopsy was a typical Type I pneumococcus in its reactions, and is designated as Strain 177-R hereafter. The possibility of intercurrent infection seemed unlikely, for

similar results were obtained with an organism taken from a blood culture, January 26. Similar organisms were obtained from blood cultures the 28th, 29th, and 30th.

Strain 177-R.—A mouse inoculated with 1 cc. of Strain 177-A, isolated January 23, 1925, died in 45 hours. The organism recovered at autopsy reacted like a typical Type I pneumococcus and was fatal for mice in 0.00001 cc. amounts. A typical Type I pneumococcus which killed mice in 0.000001 cc. doses, was obtained also from a mouse which received 1 cc. of the organisms isolated from Horse 177, January 26.

Strain 189.—Horse 189 was immunized with the standard Type I No. 5 strain, the first injection being given December 11, 1924, and the last July 31, 1925. From August 7 to August 12, the temperature of the animal ranged from 101–102.2°F. Blood cultures were sterile August 25, September 2 and 3. September 11 and September 16, a bile-soluble organism was isolated from blood cultures. It developed flocculent growth in broth and agglutinated in the three type sera, non-specifically. It did not kill a mouse in doses of 1 cc. The horse died September 17. At autopsy, the lungs were congested and hypostatic. The valves of the right heart were smooth and apparently normal but dark red in color. The bicuspid valves of the left heart were markedly thickened, and closure was incomplete. A white thrombus was attached to the auricular surface of the valve and extended through the auricle.

Strain 190.[1]—Horse 190 corresponded closely to Horse 189 as it also was immunized with the standard Type I No. 5 strain and was given first and last injections on December 11, 1924, and July 31, 1925, respectively. From August 7 to September 7, 1925, when the animal died, its temperature ranged from 101–102.6°F. Bile-soluble, Gram-positive organisms, which agglutinated in all three type sera, were isolated from blood cultures August 18 and September 3. 1 cc. failed to kill a mouse. At autopsy, an organism which gave similar reactions was isolated from the heart. The autopsy disclosed pneumonia which involved the apex of each lung and was more extensive in the right lung. There were no signs of pericarditis, but the endocarditis was marked, with extensive vegetation and destruction of the aortic and bicuspid valves.

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[1] The strains of pneumococci here recorded were also independently studied by Miss Helen R. Odell as to their virulence, type specificity, morphological and cultural reactions in the course of the examinations that are made as a part of the routine laboratory procedure. Although these tests preceded those described in this paper, the results were practically identical with the one exception that no capsules were demonstrated. Since these experiments were finished, two other atypical strains have been isolated from horses undergoing pneumococcus immunization. Both are Gram-positive, bile-soluble diplococci which agglutinate non-specifically in all three type sera. One strain, No. 150, quickly reverted to a typical Type I pneumococcus on mouse passage. The other strain, No. 201, did not kill mice in 1.0 cc. amounts.

These different strains were transferred promptly to serum semi-solid medium in which the biologic characteristics and virulence are most satisfactorily preserved for long periods of time. This medium consists of one part of 2.25 per cent agar added to five parts of serum, and has been used for this purpose since it was first described (11).

Morphology and Cultural Reactions.

All the cultures used in this study were Gram-positive cocci which grew in pairs or short chains with the typical morphology of the pneumococcus. The cultures which developed a flocculent growth in broth at the time of isolation soon formed the characteristic cloudiness of the pneumococcus when subcultured. The cultures on blood agar were all typical.

Capsules of these strains, with the exception of Strain 177-R, were demonstrated with difficulty. Those of Strain 131 were not demonstrable at all. Small capsules were shown on some organisms of the remaining strains at times, but were quite variable.

Inulin was fermented by all of the strains in subculture. The acidity of Nos. 131 and 189 exceeded that of the standard Type I culture. That of Nos. 143 and 177-R approximated the standard culture, whereas only weak reactions were obtained with Nos. 190 and 177-A.

Ox bile, one part to six parts of an 18 hour broth culture, dissolved all of these strains.

Sodium oleate 1:1000, one part to six parts of an 18 hour broth culture, dissolved Strains 131, 143, and 177-R in 30 minutes; Strains 177-A, 189, and 190 in 2 hours, at 35°C.

The vegetative activity, as compared with that of the standard Type I No. 5,[2] was studied in broth at 35°C. The usual lag (12) in the development of the freshly inoculated pneumococcus cultures was noted. This varied slightly in the different strains, but maximum growth developed with reasonable uniformity in from 6 to 7 hours. Following this, there was an interval of little or no change, but, in 24 hours, autolysis was apparent although varied in extent. This action was perhaps slightly more marked in the virulent cultures, the control

[2] Two strains of No. 5 were used for comparison; the one, a strain of exalted virulence maintained as a standard; the other, a strain which had become non-virulent as a result of repeated transfer in broth.

TABLE I.

Reactions of Atypical Pneumococci Isolated from Horses under Immunization with Pneumococcus Type I.

Strain	Source	Organism used in immunization	Capsule	Fermentation of inulin	Solubility in bile	Solubility in sodium oleate	Precipitation reaction Type sera I	II	III	131	Normal horse	Aggl. I C	I M	II C	II M	III C	III M	131 C	131 M	Normal horse C	Normal horse M	Virulence No. mice inoculated	Dose cc.	No. mice died	Phagocytosis Type sera I	II	III	131	Normal horse
131	Horse 131	I HL No. 5D	−	++	+	+	+	+	+	+	−	−	1:3200	−	1:10	−	1:200	−	1:3200	−	1:40	2 / 2	1.5 / 1.0	1 / 0	+	+	+	+	+
143	Horse 143	I HL No. 5D / I No. 5	?	+	+	+	+	+	+	+	−	−	1:3200	−	1:10	−	1:400	1:40	1:1600	−	1:40	2	1.0	0	+	+	+	+	+
177-A	Horse 177	I No. 5	? Tr.	? Tr.	+	+	+	+	+	+	−	−	1:3200	−	1:20	−	1:400	1:160	1:1600	1:2	1:40	2 / 2 / 2	1.0 / 0.5 / 0.1	2 / 1 / 0	+	+	+	+	+
177-R	Mouse inoculated with No. 177-A		+	+	+	+	+	−	−	−	−	1:20	1:160	−	−	−	−	−	−	−	−	2	0.000001	2	+	−	−	−	−

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189	Horse 189	I No. 5	?	+	+	+	+	–	–	–	+	–	1:3200	–	1:20	–	1:400	–	–	1:3200	–	1:20	–	1:40	2	1.5	1	+	+	+
																								2	1.0	0	+	+	+
190	Horse 190	I No. 5	?Tr.	+	+	–	–	+	–	–	+	–	1:3200	+	+	–	1:20	–	1:200	–	1:3200	1:2	1:40	2	1.5	1	+	+	+
																							2	1.0	0	+	+	+

Readings of precipitation and agglutination tests were made after 2 hours' incubation at 37°C. The dilutions recorded are final dilutions. The highest dilutions used were 1:3200 for the type sera and 1:40 for the normal horse serum control.

C denotes the weakest dilution in which there is complete agglutination and M the highest dilution used in which agglutination can be detected by the unaided eye.

Small, undefined capsules were at times demonstrated on some organisms of Strains 143, 177-A, 189, and 190.

The precipitation reaction in undiluted serum developed slowly and irregularly in different tests.

The agglutination reaction also developed slowly and the clumps were small.

Strain 177-A, which was more virulent for mice than were Strains 131, 143, 189, and 190, quickly reverted to a normal Type I on mouse passage, one passage in most cases being sufficient for the change. No. 177-R, the form of No. 177-A recovered from a mouse, is typical in all its reactions.

No. 5, and No. 177-R, but the differences in degree were slight, and autolysis appeared earlier and was more marked in the cultures of Strain 143, which was not virulent for mice in 1 cc. doses.

Virulence.

The virulence of all the strains was tested by the inoculation of mice and rabbits. Rats were also used in testing Strain 131.

Mice survived inoculations of 1 cc. of Strains 131, 143, 189, and 190. Of two mice receiving 0.5 cc. of Strain 177-A, one died. From it was isolated an organism which proved to be a typical Type I pneumococcus in its reactions and which killed mice in doses of 0.000001 cc. within 48 hours.

Rats and rabbits survived inoculations of from 1 cc. to 5 cc. of Strain 131 given intravenously. Rabbits also survived intravenous inoculations of 5 cc. of Strains 143, 189, and 190 and of 3 cc. of Strain 177-A.

Immunological Reactions.

In Table I the immunological and other reactions are shown.

Precipitation reactions of the clear supernatant fluids after centrifugalization of 18 hour cultures of Strains 131, 143, 177-A, 177-R, 189, and 190, were tested with horse serum of Types I, II, and III, and with No. 131 rabbit serum.

The culture broth of Strain 177-R precipitated only in Type I serum. The precipitation reaction of the other strains in undiluted serum developed very slowly. In most cases, no reaction was apparent after 30 minutes' incubation. The precipitation was irregular and incomplete except with Serum 131 which generally gave a strong reaction after 2 hours' incubation. The reaction of Strains 131, 143, and 177-A in Type III serum was often as good as, and sometimes better than, in Type I serum. Culture broth of Strains 189 and 190 precipitated only in Strain 131 serum.

Agglutination reactions with salt solution suspensions of a density equal to barium sulfate standard No. 4 from 18 hour broth cultures, developed in all the above sera with all of these strains except No. 177-R which agglutinated only in Type I serum. In most cases, the clumps were small and agglutination slow in developing. The reaction

was seldom complete except in Strain 131 serum. Agglutination occurred in much lower dilution in Type II serum than in Type I or Type III serum. Normal horse serum showed agglutination through a 1:40 dilution.

Phagocytosis of Strains 131, 143, 177-A, 189, and 190 occurred in all type sera, No. 131 serum, and normal horse serum with leucocytes of the dog. Strain 177-R was phagocyted only with Type I serum.

Precipitation tests were made with three sera produced in rabbits against Strain 131 and with supernatant broth, after centrifugalization of 18 hour broth cultures of the strains Type I No. 5, Type I HL, Type II No. 5C, and Type III No. 5A, and of Strain 131. Normal serum was used as a control. At the end of 2 hours at 37°C., Strain 131 had reacted with all three sera. After standing overnight in the cold room, all types had precipitated in the Strain 131 sera.

Agglutination was tested with organisms of Types I, II, and III, and Strain 131, and the three sera produced against No. 131. Types I, II, and III organisms agglutinated slightly in one No. 131 serum diluted 1:10. Strain 131 agglutinated in the highest dilution used of all three sera, 1:3200, but it also agglutinated in normal horse serum, 1:40, the highest dilution used.

Phagocytosis of Type I No. 5 and HL No. 5D, Type II No. 5C, and Type III No. 5A did not take place in the presence of sera produced against Strain 131.

Protection against the standard Type I No. 5 strain and Type I HL was not obtained with a serum produced against Strain 131.

Soluble Specific Substances of Strain 131.

The extent of the attenuation of these pneumococci, together with their irregular serologic reactions in immune and in normal serum, suggested a study of their specific soluble substances in comparison with those of the standard Type I strain. This was undertaken by Dr. Anthony Kozlowski, but the work was interrupted by his return to Poland.

The observations of Heidelberger and Avery (13, 14) were confirmed in that the specific soluble substance described by them was obtained from the standard Type I Strain 5. From this attenuated Type I strain (No. 131) substances were obtained which reacted specifically

not only in Type I serum but also in Type III serum; also, substances which precipitated non-specifically in all three type sera and in serum produced against Strain 131.

Although quite large amounts of Strain 131 organisms were used, and numerous attempts made, it was not possible, with identical methods, to isolate a sufficient amount of substance, which would react with only one type serum and be entirely free from protein derivatives, to establish its chemical character. It is hoped that further studies will indicate more definitely the nature of the chemical changes in the specific substances which are associated with the attenuation of the culture. For the present it is important to note, however, that the production of the specific soluble substances was altered in the attenuated cultures.

SUMMARY.

It has been shown that the pneumococcus multiplying in the tissues of the immunized animal (horse) becomes attenuated: loses, in varying degrees, its virulence, capacity of capsule formation, susceptibility to phagocytosis, and type specificity. The antigenic activity as an immunizing agent and the production of "soluble specific substance" are also altered. In some instances, the typical pneumococcus characteristics may be quickly restored by one or two passages through a susceptible animal (mouse). In others, virulence is not recovered and the organism remains atypical.

Whether these changes are to be attributed to the specific action of immune bodies in the tissues, or are to be considered as the result of some biologic adaptive process to an adverse environment, has not been determined. Proof of the specific action of immune bodies is possibly open to question, whereas it is well known that virulence and with it some other characteristics are profoundly affected under unfavorable conditions in the absence of immune bodies, notably when the organism is grown at elevated temperatures or in certain unsuitable media.

These studies of pneumococci isolated from the infected immunized horse provide opportunities for further investigation of the significance of changes in virulence, type specificity, and formation of "soluble specific substances" in various forms of pneumococcus infection.

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COMPARISON OF A VIRUS OBTAINED BY KOBAYASHI FROM CASES OF EPIDEMIC ENCEPHALITIS WITH THE VIRUS OF RABIES.

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In 1924 there was a sudden outbreak of a form of epidemic encephalitis in Japan with a reported mortality rate of over 50 per cent which resulted in more than 4,000 deaths. During the epidemic Dr. Kobayashi[1] succeeded, after many attempts, in isolating from a single case of the disease a "virus" capable of indefinite transmission in experimental animals and very courteously sent samples preserved in glycerol to Dr. Flexner for study.

This virus is of peculiar interest. In the first place, because it is doubtful whether previous attempts to transmit the virus of epidemic encephalitis to animals have ever been successful.[2] Secondly, on account of the possibility that the virus of Japanese encephalitis may be of a different kind which, perhaps, may be more easily transmitted than the others, and be thus more readily studied.

The virus was isolated by Kobayashi through the subdural inoculation of rabbits with brain substance from a typical case of epidemic encephalitis diagnosed clinically as well as post mortem. The first symptoms were noted in rabbits on the third passage and subsequently in almost 100 per cent of rabbits inoculated subdurally or corneally. In his opinion, it resembles closely the virus of Koritschoner[3] and the virus isolated from dogs by Hoff and Silberstein.[4] He compared it with the fixed virus of rabies and found many and significant points of similarity as well as the following differences:

1. The encephalitis virus occurred, for the most part, in the brain and was not

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[1] Kobayashi, R., *Japan Med. World*, 1925, v, 145.

[2] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[3] Koritschoner, R., *Virchows Arch. path. Anat.*, 1925, cclv, 172.

[4] Hoff, H., and Silberstein, F., *Z. ges. exp. Med.*, 1924–25, xliv, 257, 268.

demonstrated in the spinal cord or medulla; whereas that of rabies was found chiefly in the spinal cord.

2. When the encephalitis virus was inoculated on the cornea of 6 dogs, none developed the disease; but when 5 were inoculated subdurally, 4 were affected. With the virus of rabies, on the other hand, 2 out of 3 dogs developed characteristic symptoms after corneal inoculation and all after subdural inoculation.

3. The course of the disease produced by the encephalitis virus was prolonged, and that caused by the virus of rabies, acute.

Evidently it is important to extend the researches of Kobayashi to determine if possible the exact nature of his virus. The following immunological study was therefore made of the encephalitis virus which he sent us and of a fixed rabies virus obtained through the courtesy of Dr. Anna Williams of the New York City Department of Health.

EXPERIMENTS.

In order to test the cross-immunity, if any, existing between the encephalitis virus and the virus of rabies a procedure suggested by Dr. Williams' assistant, Mr. Tyler, was employed with slight modifications.[5]

The viruses were inoculated intracerebrally into a series of young rabbits. The brains of the animals which showed the most characteristic symptoms were used for the preparation of standard viruses of both kinds, which were preserved in ampules of non-alkaline glass. The M.L.D. were then ascertained by the intracerebral inoculation of guinea pigs weighing about 250 gm. These proved to be the same for both viruses, namely $\frac{1}{15,000}$ gm. of brain substance, although the incubation period was uniformly longer in the case of the encephalitis virus, which, on intramuscular injection, was much less potent than the rabies virus. For the first cross-immunity studies two series of 20 guinea pigs of approximately the same weight (250 gm.) were selected.

Series A was given a Pasteur treatment, consisting of 20 daily subcutaneous injections of inactivated fixed rabies virus. Inactivation was effected by making up an 8 per cent suspension of brain substance in 1 per cent carbolic acid in saline solution, and by incubating this at 37°C. for 24 hours. This was diluted with equal parts of sterile saline and, just before use, was diluted again 1 to 3. The amount injected was 0.5 cc. The "vaccine" was tested and found to be free from contaminating bacteria.

Series B received Pasteur treatment in the same way with the inactivated encephalitis virus.

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[5] Help received from Mr. Peter Haselbauer was invaluable.

The animals were left for 1 week after the conclusion of treatment before testing for cross-immunity. The injections were all made intracerebrally and the injected viruses were tested for bacterial contamination and found to be free from it. The symptoms of those succumbing were identical following the action of both viruses. After an incubation period of about 3 days, the temperature rose to about 105°F. on the 4th or 5th day. The animals then showed excessive salivation, tremors, convulsions and paralyses. The tem-

TABLE I.

The Action of Encephalitis Virus upon Animals after Pasteur Treatment with Rabies Vaccine.

Experiment	Control	Control	Control
Pasteur treatment with fixed rabies vaccine + encephalitis virus	Pasteur treatment with fixed rabies vaccine + fixed rabies virus	Untreated normal animals + encephalitis virus	Untreated normal animals + fixed rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
No characteristic symptoms or deaths	2 animals receiving 2 M.L.D. developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died

perature commenced to fall on the 6th or 7th day and became rapidly subnormal—94° or 95°F.—when the animals died.

An examination of the results which are set forth in Table I shows:

1. That six animals which received Pasteur treatment with the rabies virus resisted subsequent inoculation with the encephalitis virus even to the amount of 3 M.L.D.

2. That the Pasteur treatment with the rabies virus produced a measure of immunity against the rabies virus itself.

3. That untreated normal animals generally succumb to the action of the encephalitis virus showing that the sample of encephalitis

TABLE II.

The Action of Fixed Rabies Virus upon Animals after Pasteur Treatment with Encephalitis Vaccine.

Experiment Pasteur treatment with encephalitis vaccine + fixed rabies virus	Control Pasteur treatment with encephalitis vaccine + encephalitis virus	Control Untreated normal animals + fixed rabies virus	Control Untreated normal animals + encephalitis virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
2 animals 4 M.L.D. virus	2 animals 4 M.L.D. virus	1 animal 4 M.L.D. virus	1 animal 4 M.L.D. virus
One 1 M.L.D. animal died of some cause unknown 11 days after injection	One 2 M.L.D. animal died from peritonitis resulting from intestinal perforation. The others were unaffected	One 1 M.L.D. animal was found dead, no characteristic symptoms having been noted	One 3 M.L.D. animal developed characteristic symptoms and died
One 2 M.L.D. animal developed characteristic symptoms and died			
One 3 M.L.D. animal developed characteristic symptoms and died			
Two 4 M.L.D. animals developed characteristic symptoms and died			

virus has retained its potency. The M.L.D. originally determined is too low because during the experiments, which extended over a period

of more than a month, the animals gained on the average 50 gm. in weight, in consequence of which 1 M.L.D. was not effective.

4. That untreated normal animals likewise succumb to the action of the fixed rabies virus indicating, in the same way, that it likewise has retained its potency. The fact that 1 M.L.D. was not effective is to be explained on the basis above mentioned of the increase in weight of the animals.

TABLE III.

The Action of Fixed Rabies Virus upon Animals 6 Days after Last Immunizing Dose with "A" and "B" Encephalitis Vaccines.

Experiment Pasteur treatment with encephalitis vaccine "A" + rabies virus	Experiment Pasteur treatment with encephalitis vaccine "B" + rabies virus	Control Untreated normal animals + rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus
One 3 M.L.D. animal died with typical symptoms*	All recovered but one had slight tremors	The animals receiving 1 and 3 M.L.D. died with typical symptoms.* The animal receiving 2 M.L.D. exhibited tremors, and became ataxic, but recovered

* Examined histologically.

It seems clear, therefore, that the action of the encephalitis virus was inhibited by Pasteur treatment with rabies vaccine so that a one-sided immunity exists.

The observations in Table II were designed to show whether the converse also holds. They are arranged in the same manner, but are less concise, because only 3 out of 8 animals, which received the Pasteur treatment with the encephalitis virus, resisted inoculation with the rabies virus. The controls are similar.

A further attempt was then made, along slightly different lines, to answer the same question as to whether it is possible to protect

animals against the rabies virus by Pasteur treatment with the encephalitis virus.

Instead of making one encephalitis "vaccine," as in the earlier experiments, two were made (designated "A" and "B") each from the brain of a typical case of the disease in a rabbit. The dose of vaccine was increased from 0.5 cc., used in the previous experiments,

TABLE IV.

The Action of Rabies Virus 9 Days after Pasteur Treatment with Encephalitis Vaccines "A" and "B."

Experiment Treated with vaccine "A" + rabies virus	Experiment Treated with vaccine "B" + rabies virus	Control Treated with vaccine "B" + encephalitis virus	Control Normal untreated + rabies virus	Control Normal untreated + encephalitis virus
2 animals received 1 M.L.D. virus	2 animals received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus
2 animals received 2 M.L.D. virus	2 animals received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus
2 animals received 3 M.L.D. virus	2 animals received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus
The 2 M.L.D. animal died with typical symptoms*	No characteristic symptoms: all recovered	No characteristic symptoms: all recovered	All died with typical symptoms*	The 1 M.L.D. animal developed tremors but recovered: the others died with typical symptoms*

* Examined histologically.

to 0.6 cc. The M.L.D. were so calculated as to apply to the animals after the period of immunization when each had gained about 50 gm. in weight. Both vaccines were employed in two sets of experiments and each set of animals thus treated was tested for immunity, respectively, 6 and 9 days after the last immunizing dose.

The results of the first set are detailed in Table III and it is evident that both encephalitis "vaccines" conferred a high degree of

immunity against the action of the rabies virus. The second set of experiments, recorded in Table IV, seems entirely to confirm this result. In other words, there is reason to believe that cross-immunity is demonstrable between the virus isolated by Kobayashi from a case of epidemic encephalitis and the fixed virus of rabies.

Although the immunological properties of the two viruses are thus apparently identical the action of the rabies virus is more rapid and on intramuscular injection more fatal. For example, 10 guinea pigs, which received intramuscularly at the back of the neck 0.5 cc. of a 10 per cent brain emulsion containing rabies virus, all developed characteristic symptoms and died. Whereas 10 other guinea pigs of about the same size which received similar doses of the encephalitis virus all lived. Differences of this kind may presumably be explained on the supposition that the two viruses, though of the same nature, possess different degrees of virulence.

Histological Studies.

The brains of 9 animals (marked with an asterisk in the tables) were examined after fixation in Zenker's fluid and coloration with Giemsa's stain. It was found that the lesions caused by the encephalitis virus and the rabies virus were identical.[6] Similarly 2 young dogs injected intracerebrally with the encephalitis virus developed symptoms indistinguishable from the familiar symptoms of

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[6] Some of the rabbits employed exhibited lesions occasioned by another and wholly different disease, namely, spontaneous encephalitis. These lesions, however, do not constitute a source of error, once their existence is recognized, because they are of slow development and are sometimes associated with a specific and easily recognizable parasite.

In this connection it is desirable to supplement a foot-note in a recent paper on the geographic distribution of spontaneous encephalitis (Cowdry, E. V., J. Exp. Med., 1926, xliii, 730). The note is as follows: "In addition, twenty-four Swedish rabbits, which had been sent to Dr. Flexner by Dr. Kling of Stockholm, were thoroughly examined. Two showed encephalitis and nephritis; one, encephalitis only, and another, only nephritis. No encephalitozoa were observed." It should be added that: "seventeen of these rabbits had been inoculated with brain substance sent by him to us, that the lesions mentioned were restricted to these inoculated animals and that the remaining seven rabbits were employed for control purposes and showed neither symptoms nor lesions."

furious rabies and died. In order to make sure of the comparison a third dog received an intracerebral injection of the street rabies virus and behaved in precisely the same way. Large and typical inclusion bodies were observed by a variety of methods in the brains of all 3 dogs.

CONCLUSION.

Since the symptoms produced in experimental animals by the encephalitic virus of Kobayashi and by the virus of rabies are similar, and are accompanied by lesions which are indistinguishable, and since a cross-immunity is demonstrable between the two viruses, the conclusion is advanced that the specimen of so called encephalitis virus isolated by Kobayashi is in reality a specimen of rabic virus.

THE OCCURRENCE OF DEGRADED PNEUMOCOCCI
IN VIVO.

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It has been well demonstrated that under certain experimental conditions *in vitro* pneumococci undergo variations. It is of great importance to determine whether variants ever appear *in vivo* and if so, under what conditions. This is especially important since several investigations have suggested that recovery from pneumococcus infection may depend largely upon a change of virulent pneumococci into avirulent and phagocytable forms. This paper describes attempts to determine the experimental conditions favoring a change of virulent (S) pneumococci into avirulent (R) forms within the animal body.

It has been repeatedly shown (1–3) that by subjecting type-specific (S) pneumococcus strains to various unfavorable cultural environments *in vitro*, certain biological characteristics of the bacteria are profoundly affected. The changed bacteria no longer elaborate the specific carbohydrate element upon which type specificity depends and they are therefore agglutinable in heterologous as well as homologous antipneumococcus serum (4), they are no longer virulent for mice, and they are easily phagocyted (5, 6). These organisms have been designated R pneumococci by Griffith on account of the rough surface of the colonies which they form when grown on solid media, in contradistinction to the smooth surfaced colonies formed by S or typical virulent pneumococci. This difference renders the two forms distinguishable microscopically and often macroscopically when colonies of both forms are present on the same blood agar plate.

An intermediate form which is somewhat less virulent than the original S strain and also possesses slight serological differences has also been described (7). By animal passage it is possible to restore the original virulence and type specificity of this form. On the other hand, by placing it under unfavorable cultural environment the intermediate

form is easily converted into the R form. It would seem that the intermediate organisms represent a transitional stage between the S and the R forms.

Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy. But in case the change from S to R does take place *in vivo* it seems probable that R forms would be phagocyted and destroyed as soon as they appear. Therefore, to determine the presence of the R forms *in vivo*, it would probably be necessary to employ a technic under which the infection would remain localized over a considerable period of time without killing the animal, and under which the free access of phagocytes to the bacteria would be inhibited. It was thought that these conditions might be obtained by embedding subcutaneously in the experimental animal an agar mass inoculated with a virulent strain of Pneumococcus.

Method.—15 cc. of melted nutrient agar was inoculated with 0.5 cc. of a young broth culture of Pneumococcus. A wire loop about 4 cm. in diameter was pressed firmly over a shaved area of skin of the anesthetized animal and the fluid agar injected subcutaneously into the area enclosed by the loop. Ice was applied until the agar had solidified into a firm hemispherical nodule. Observations were made from time to time by puncturing the focus with a hypodermic needle, aspirating some of the material, and plating it on blood agar plates. The plates were examined for S, R, and intermediate colonies after 12–18 hours incubation.

Experimental.

Agar Foci in Dogs.—Dogs were first employed since these animals have a natural relative immunity to Pneumococcus, and were likely to withstand infection over a considerable period of time. Two dogs were injected with agar containing Type I pneumococci according to the method described. Material was aspirated from the foci at intervals from the 1st to the 14th day after injection. But R forms never appeared on the culture plates made from the material at any time, although S forms were constantly present in large numbers. The dogs soon became ill and S pneumococci were recovered from the blood stream on several occasions. In a few days abscesses formed at the site of injection in both animals and by the 10th day the skin

over the foci ruptured and thick pus was discharged. About the 14th day the lesions began to heal, the pneumococci disappeared and the dogs recovered. After a rest period of 2 weeks the same dogs, which were now assumed to have an additional degree of immunity, although specific agglutinins were not demonstrable in the blood serum, were reinjected with infected agar. A repetition of the previous events occurred. The dogs recovered and no R forms were encountered either from the foci or from the blood stream. While it was impossible with this technic to demonstrate the appearance of R forms in dogs, it should be mentioned that 11 years ago Bull (8) discovered that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. In one dog which lived for 10 days before dying with meningitis, pneumococci isolated from the blood on the 9th day "grew in chains and were non-virulent."

Agar Foci in Rabbits.—The same technic was then employed with rabbits instead of dogs, and instead of using a Type I strain, a Type III strain was employed. This type was used because rabbits have a high degree of resistance to Type III pneumococci, in contrast to their susceptibility to Types I and II, and it was thought that animals infected with this type would live longer and the bacteria would therefore have a greater chance to undergo variations. The special strains employed have been shown to readily undergo variations *in vitro*. Three rabbits were used. Daily examination of the foci by aspirating and plating material from the foci on blood agar plates showed that only S forms were present. The number of pneumococci gradually diminished until no more were demonstrable by the 6th day after injection.

Agar Foci in Guinea Pigs.—In a similar manner six guinea pigs, actively immunized against Type I pneumococci, were injected with agar containing Type I pneumococci. Repeated cultures from the foci showed the presence of S forms alone until the 5th day after injection when eight R colonies were found on a blood agar plate seeded from the focus of one guinea pig. R colonies were subsequently recovered from the foci of the other five guinea pigs at intervals up to 6 weeks after injection. In all instances however, the S colonies greatly outnumbered the R forms and were recovered from the foci for 6 weeks, or until the agar was absorbed. Normal (unimmunized)

guinea pigs were also tested but died from pneumococcus septicemia too soon to be suitable for the experiment.

In spite of the mass of agar surrounding the pneumococci in the subcutaneous foci, the object of preventing the access of phagocytes was not attained. Stained films made from the aspirated agar showed the constant presence throughout the agar mass of many phagocytes containing pneumococci.

As mentioned above, it was assumed that the R forms, being easily phagocyted, were rapidly destroyed or removed from the site of infection. To test this assumption, agar masses inoculated with a heavy suspension of R pneumococci were injected subcutaneously into two guinea pigs. It was found that the R forms were recoverable for 5 weeks after injection or until the foci had been completely absorbed. Although microscopic examination revealed the presence of great numbers of phagocytes, they apparently had but little influence in removing the R forms as long as the focus remained.

In order to eliminate the activity of phagocytes entirely, the technic was then modified as follows: Agar, which was inoculated with S pneumococci, was enclosed in a glass vial sealed with a collodion membrane. The membrane was an effective barrier against phagocytes but permitted the diffusion of fluids.

Method.—5 cc. wide mouth vials were filled, nearly to the top, with melted agar and the mouths were covered with thin muslin caps. After sterilization the agar was inoculated with 0.1 cc. of pneumococcus broth culture by means of a long hypodermic needle, and the muslin-covered mouths were dipped in a thin solution of collodion. The vials were then immersed in 95 per cent alcohol for several minutes to render the membrane more permeable. The sealed vials were embedded subcutaneously in rabbits. Repeated observations were made by inserting a long hypodermic needle through the skin of the animal and the membrane of the vial, aspirating some of the agar, and plating it on blood agar.

Control vials were prepared in the same manner but, instead of being placed *in vivo*, they were immersed in normal rabbit serum in large test-tubes. The tubes were kept in the incubator at 39°C. which was considered to be the average temperature of the vials while embedded in the rabbits. The serum was changed weekly.

Vials containing agar inoculated with cultures derived from a single diplococcus of a Type I strain were inserted subcutaneously in eight normal and three passively immunized rabbits and allowed to remain

for several weeks. Eight vials similarly prepared were immersed in normal serum and kept in the incubator as controls. Agar was aspirated from the vials and plated on blood agar at intervals of from 1 to 5 days. Several of the normal (unimmunized) rabbits soon died from pneumococcus septicemia due to infection resulting from leakage of the vials after aspiration. The unimmunized rabbits which survived were observed over a period of 8 weeks. Their serum did not show the presence of agglutinins for Type I pneumococci even at the end of this time.

R colonies were invariably found on plates made from all of the vials embedded in rabbits. They appeared, often with the intermediate forms, as early as the 3rd day after inoculation and were recoverable up to 8 weeks. The variant colonies were usually outnumbered by the S forms and in no instance were the S forms entirely replaced by them. It was rather surprising to find that both R and intermediate colonies were derived from the agar vials in normal and immunized animals alike.

The eight control vials inoculated at the same time with the same culture of Type I pneumococci were kept in the incubator. R colonies never appeared on plates made from these vials although the S forms remained during the period of observation.

Experiment with Type III Pneumococcus.—Three agar vials were inoculated with a Type III pneumococcus culture obtained from a single diplococcus from the blood culture of a pneumonia patient, and embedded subcutaneously in three rabbits. Frequent plating of the agar at intervals during a period of 5 weeks never revealed the presence of R or intermediate forms although S forms remained viable throughout.

This culture proved to be a strain especially refractory to modification as was determined by subjecting it to treatment *in vitro* by methods which invariably caused the appearance of R forms in other strains. Frequent search during 40 transfers in broth containing 5 per cent Type III antipneumococcus serum, or during 11 transfers in broth containing optochin yeast (9), or during 53 transfers on blood agar plates, did not reveal the presence of any R or intermediate forms. The culture remained unchanged in virulence and type specificity. Takami (10) has also encountered resistant strains of this

nature. He found that, of 28 strains, 12 never gave rise to variant forms even after prolonged subculture on blood agar.

Spontaneous Appearance of R Forms in Vivo.—Aside from the experimental production of the variant forms it is of interest to report the spontaneous occurrence of R forms *in vivo*. Through the courtesy of Dr. Mary B. Kirkbride of the New York State Department of Health, it was learned that atypical pneumococci were occasionally recovered from the blood stream of horses during the process of immunization with live virulent cultures for the production of antipneumococcus serum. The atypical strains were recovered from 6 to 16 months after the horses were first inoculated with a Type I pneumococcus culture. During this time the horses had a low grade fever, anorexia, loss of weight, rapid irregular pulse, heart murmurs, swelling and stiffness of the joints. The animals finally died and autopsy revealed an endocarditis present in all. Atypical pneumococcus strains from four of these horses were obtained and examined in this laboratory. They proved to have many characteristics in common with the variant or R strains previously studied. Each of the four strains produced colonies which differed slightly morphologically from one another and were distinguishable on blood agar plates. It was previously noted that in a number of instances, R colonies differing from one another in appearance were also recovered from the agar vials embedded in rabbits. The four horse R strains together with four strains derived from R colonies from the rabbit vials were tested for variations in virulence and agglutinability. All strains failed to kill mice in doses of 1 cc. of a young broth culture, were bile-soluble, and were serologically alike. The morphological colony differences between the various strains persisted even after repeated plating. Repeated (six) animal passages of the R strains failed to restore either virulence or specificity.

DISCUSSION AND CONCLUSION.

It is conceivable that a change from the virulent, non-phagocytable S form of Pneumococcus to the avirulent phagocytable R form may take place in pneumococcus disease, but the experiments here reported do not settle the question whether or not this is an important factor in determining the outcome in natural infection. It has been shown

experimentally that the degradation from the S form to the R form actually does take place in cultures of Pneumococcus growing in agar subcutaneously embedded in guinea pigs, in agar enclosed in vials subcutaneously embedded in rabbits, and spontaneously in the blood stream of infected horses. However, it was not possible in any of the experiments here cited to demonstrate the complete change from S to R pneumococci before the bacteria disappeared from the body. When the intermediate or R forms did appear, they were always accompanied and usually exceeded in number by the S forms and all three forms disappeared together. S organisms may disappear entirely without evidence of first going through the intermediate and R stages. On the other hand, contrary to expectations, pure cultures of R forms remained viable in subcutaneous foci for weeks although apparently freely accessible to the action of phagocytes. It seems of some significance that the R forms appeared early in the vials (inoculated with S pneumococci) in immunized and normal rabbits alike, indicating that the presence of demonstrable specific immune bodies was not alone responsible for the variation of the bacteria.

Of some importance also is the fact that R forms were never derived from similarly prepared control cultures growing *in vitro* at the same temperature and immersed in normal serum, although the S forms remained viable and unaltered for 6 weeks. It is likely that variations of pneumococci do not occur readily when S cultures are exposed to normal serum *in vitro*, especially when growing in closed vials under a diminished oxygen supply, for it has previously been shown (2) that only slight variation occurs even after prolonged (240) transfers in heterologous serum broth in the test-tube. It is possible, therefore, that the variation which occurred among pneumococci growing in agar vials embedded in normal rabbits was actually provoked by unknown influences in the living tissue fluids.

Although R forms have been shown to occur *in vivo*, no positive evidence can be derived from these experiments to prove that recovery from pneumococcus infection depends upon the degradation of the virulent S forms of pneumococci to the avirulent R forms and the subsequent destruction of the latter by phagocytes.

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STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

III. RESISTANCE TO CHEMICALS.

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Reference has already been made to the minuteness of size of the virus of foot-and-mouth disease and to its electropositive charge in the first two papers of this series.[1,2] We believe that these two factors serve to explain in part the unusual resistance of the virus to certain chemicals. So great is its tenacity in the presence of strong reagents that one might interpret this phenomenon as a sign of the inanimate character of the incitant. But as the sequel will show these chemical actions are illusory.

Resistance to Chemicals.

Although filter-passing viruses are generally resistant to certain chemicals, the active agent of foot-and-mouth disease is exceptionally so. In this remarkable resistance a parallel has been found thus far in the virus of mosaic disease.

For example, Abe[3] found that 70 to 75 per cent alcohol precipitates the virus of foot-and-mouth disease, along with the albumens of infective blood, which can then retain its activity for 2 to 3 days in a dried state. Stockman and Minett[4] reported that 25 to 50 per cent alcohol does not destroy it after at least 3 days and 10 per cent, after at least 20 days. With regard to chloroform and ether, these writers state that 10 drops of chloroform to 5 cc. of 1:100 virus do not inhibit activity after at least 27 days; and 10 drops of ether to 3 cc. of filtrate do not destroy

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[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

[2] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 685.

[3] Abe, T., *Z. Infektionskrankh. Haustiere*, 1925, xxviii, 111.

[4] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

it after more than 10 days. In similar proportions of acetone the active agent survives for at least 4 days, and in 50 per cent glycerol, as is the case with many filter-passing viruses, indefinitely. These remarkable conditions have also been confirmed in general by Bedson and Maitland.[5]

The results of our experiments are in agreement with those of Abe and the British investigators. For we have found repeatedly that in 20, 40, 50, and 60 per cent alcohol the virus either in ground infected pad tissue or in aspirated lymph remained alive for at least 26 hours. In 5 experiments, the virus admixed with practically undiluted acetone maintained its viability for 20, but not for 30 minutes. Nor was it killed by supersaturation with ammonium sulfate—the active agent was viable in the coagulum for at least 2 hours.

In view of the fact that bacteria generally are destroyed very rapidly by these agents (staphylococci in 1 minute by 60 per cent alcohol), we believed that it was sufficiently important to undertake a comprehensive series of experiments which might indicate whether the resistance to chemicals was real or merely masked. We were aware that under the conditions employed by earlier investigators, a comparison between the resistance of the virus and that of bacteria, such as staphylococci, could not be made. In the case of the latter the reagents were added to a pure culture or a material which was presumed to contain nothing but bacteria and the diluent; in that of the virus the chemicals were added to tissues or the solid and fluid constituents, mainly protein, of an inflammatory exudate, of which the virus occupied only a small part. Furthermore the chemicals to which the active agent is resistant are all capable of coagulating proteins. That the virus is readily adsorbed to these coagula will be shown in the following protocol.

Protocol 1. Control.—Infected guinea pig pads 24 hours after injection were ground with sand and a quantity of 90 per cent alcohol was added to make the final dilution of the latter 60 per cent. A heavy coagulum resulted. After 2 hours centrifugation at 2500 R.P.M., the clear supernatant fluid and the sedimented material were injected intradermally in 3 guinea pigs, respectively, for each of the 2 portions. After 24 hours all 6 showed typical primary lesions and after 48 hours, secondary vesicles in the mouth and pads.

[5] Bedson, S. P., and Maitland, H. B.. *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

Test.—At the same time, the coagulum of similar alcoholized material, which was active for at least 26 hours, was allowed to settle for 1 hour. The topmost and lowest portions were injected into guinea pigs with the result that all 4 animals exhibited primary lesions 24 hours after inoculation, and secondary lesions 24 hours later. The supernatant fluid was filtered through Berkefeld candles. The filtrate was free from virus.

This experiment is typical of 7 others. In the remaining tests the contact with alcohol before filtration covered a period of from 2 to 3 hours and the virus employed consisted of infective blood, ground pad tissue, or aspirated lymph.

To summarize the results of these tests it may be stated that alcohol, when added to blood, ground pad tissues, or aspirated lymph containing the virus, causes the formation of a more or less dense coagulum. Centrifugation of these agglomerations leaves a supernatant fluid which is still active. We have already shown that the virus as it occurs in the tissues of the guinea pig is not centrifugable and we have discussed the inability of the centrifuge to depose or sediment completely minute particles.[1] On the other hand, filtration is a more effective means of separating particles from a fluid. Hence by employing Berkefeld filters, which owing to their relatively larger sized pores permit the passage of the active agent irrespective of electric charge,[2] these particles are thereby withheld from the filtrate. The filtrate then is free from virus. In other words, the active agent is closely attached or adsorbed to or forms a nucleus in the center of the coagula formed after the addition of alcohol.

Similar results were obtained with acetone. In pure acetone, which forms a heavy soft coagulum, the active agent survived for 20 but not for 30 minutes. If the material was filtered immediately after complete coagulation occurred, the Berkefeld filtrate was inactive.

With ammonium sulfate the result was the same. In this instance, materials containing the virus after supersaturation with ammonium sulfate for 1 hour at 37°C., exhibited a heavy coagulum. The latter was active but on filtration of the substance through Berkefeld candles, the filtrates were inactive. In two instances the clear filtrate obtained by employing 2 layers of Schleicher and Schüll, No. 589 filter paper was also free from virus.

Hence it appears that the active agent in guinea pig tissues is completely adsorbed, and protected, as we shall soon show again,

by the precipitates formed after the addition of alcohol or acetone or ammonium sulfate.

The next step to suggest itself in a further study of this phenomenon was the relation of the amount of protein in the material containing the virus to its capacity for resisting the action of the reagents.

By filtration of vesicular fluid through Berkefeld candles, it was possible to remove a considerable amount of protein substances, especially cells, fibrin, clots, and larger miscellaneous particles. Filtrates, however, usually showed the biuret reaction so that not all proteins were thus eliminated. However, when alcohol or acetone was added to filtrates, only a faint haze of micellæ resulted. How this influences the resistance of the virus to the reagents is illustrated in the following experiment.

Protocol 2.—1 cc. of aspirated lymph from pads injected 24 hours previously was added to 20 cc. phosphate buffer at pH $= 7.5$. This was then filtered through a Berkefeld N candle and of the filtrate 5.4 cc. were taken. To this amount, 10 cc. of 90 per cent alcohol were added which made the concentration of the latter equal to 60 per cent. 0.1 cc. of the remainder of the filtrate, non-alcoholized, was injected into each of 2 guinea pigs, as controls, to test its activity. In 48 hours both animals exhibited the typical experimental disease.

A series of guinea pigs was now inoculated intradermally with the alcoholized filtrate after varying intervals: After 5 minutes, only 1 of 2 animals injected was positive with an incubation period of 4 days; after 15 minutes the same result occurred with an incubation of 5 days; after 30 minutes and 1 hour, the filtrate proved inactive.

This experiment is typical of several others in some of which 50 per cent alcohol as well as filtrates of ground infected pad tissues were also employed.

The conclusions to be derived from all these tests are that although the unfiltered virus remained viable in 50 or 60 per cent alcohol for at least 26 hours, filtered material showed no such resistance: When 50 per cent alcohol was added to active filtrates of aspirated lymph or ground infected pad tissues, the virus was killed within 15 to 20 minutes, and in 60 per cent alcohol, within 1 to 15 minutes, depending on the concentration of the active agent. The unfiltered material contained more protein substance than the filtered and hence a greater amount of coagulum which displayed a greater ad-

sorptive function.   The consequence was that the virus in filtrates was more open to the direct attack by the reagent.

As a corollary, the immediate refiltration of the active coagulum formed by adding alcohol to filtrates, removed the virus.   This is not due to double, or repeated, filtration of the virus materials, for control tests showed no such tendency.

From these observations further proof is adduced that there is a relationship between the amount of virus adsorbed to large coagula and the killing effect of alcohol.   There is an indication here which points to the possibility of increasing the destructive action of alcohol, if the latter could be placed in contact with free, or unadsorbed, virus.   We have already stated[1] that the virus can exist in a free state.

We believe that we have succeeded in bringing this about by taking into consideration the fact that coagulation of proteins by alcohol is a periodic phenomenon, depending on hydrogen ion concentration.[6,7] For example, a basic experiment was performed with ascitic fluid as the protein material.   It was found that the heaviest coagulation by 60 per cent alcohol occurred between pH = 7 and 7.6, maximum at pH = 7.5.   At pH = 8.2 there was absolute clearing and at pH = 6.5, only a slight micellar haze was noted.   Hence at a certain point, reached by adding a definite minute quantity of 0.5 per cent NaOH to protein material, alcohol does not coagulate.   Furthermore, the quantity of alkali necessary to prevent this coagulation is insufficient *per se* to kill the virus.   Since it is impossible to make determinations of the hydrogen ion concentration of alcohol, we proceeded in an empiric manner, after the method of Loeb[8] in his studies on solutions of protein in alcohol-water mixtures, to determine precisely the amount of alkali necessary to inhibit coagulation.

Protocol 3.—A large number of experiments was made over a period of 3 months and several hundred guinea pigs were employed before unequivocal results could be

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[6] Compare Michaelis, L., Manuel de techniques de physico-chemie, translation by Chabainer, H., and Labo-Onell, C., Paris, 1923, 81 ff., or the original in German.

[7] Kopaczewski, W., Théorie et pratique des colloïdes, Paris, 1923.

[8] Loeb, J., Proteins and the theory of colloidal behavior, New York, 2nd edition, 1924, 359 ff.

obtained. It is only possible to state here a résumé of results, and of the conditions necessary for procedure.

We found these technical operations to be important in carrying out the tests.

1. All material should be measured in conical beakers or chemical mixing glasses and not in test-tubes, for spattering of the virus or insufficient mixing of the substances may give irregular results.

2. The active virus, whether in aspirated lymph or ground infected pad tissues should be filtered first through filter paper to remove clots and small particles. The dilution employed in tests and controls was 1:50 to 1:100. In each experiment, the paper filtrate was injected intradermally in guinea pigs to test its activity.

3. The phosphate buffer[9] at pH = 7.5 should be adjusted to this point just before use, since sterilization or standing in glass utensils may cause a change in hydrogen ion concentration. The buffer is used only for making suspensions of the virus. For controls (see Table I) distilled water is substituted for the alcohol or NaOH as the case requires so that the buffer effect remains constant throughout each series.

4. In the test, the alkali should be added first, then the alcohol (for water in controls), and lastly the virus. Immediately thereafter these ingredients are thoroughly mixed, and after the time set for contact, fluid is removed from about the centre of the mixture.

5. Since the measure of the killing effect of alcohol is not cultural but biological, that is, the injection of the test materials intradermally in guinea pigs, great care should be observed in differentiating the dull white alcoholic, or the dirty yellow alkali, inoculation necroses, from the lesions of experimental foot-and-mouth disease. As safeguards it was necessary, (a) to have a strain of virus which produced regularly secondary vesicles, (b) to transfer suspected tissues to normal guinea pigs for the observation of uncomplicated effects, and (c) whenever possible to subject recovered animals to an immunity test.

In the tests 60 per cent, 40 per cent, and 20 per cent alcohol dilutions were employed. These percentages indicate the final concentrations which were obtained by using stock alcohol of 90 per cent strength. Of this a sufficient amount was taken to make the required concentration, in a total volume of test or control materials of 7.7 cc.

It was found that in this volume and with these percentages of alcohol the quantity required of the NaOH, 0.5 per cent solution, was 0.33 cc.

In Table I, the different amounts of the materials employed are tabulated, as well as the general manner of procedure, both in regard to the tests and their corresponding controls.

From this tabulated résumé it is to be noted that the active agent as it exists in the tissues or exudates of the guinea pig resists the action

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[9] For its preparation, Papers I and II should be consulted.

of alcohol in dilutions of 60, 40, or 20 per cent.   But the resistance is due to the adsorbing coagulum which results from the interaction of alcohol on the proteins of the milieu in which the virus resides.   The latter is also unharmed by the presence of NaOH 0.5 per cent in the

TABLE I.

Effect of Alcohol on Virus.

Time	For 60 per cent proportion			For 40 per cent proportion			For 20 per cent proportion		
	Control	Control	Test	Control	Control	Test	Control	Control	Test
	Virus 2.7 cc. + alcohol 90 per cent 5 cc. + $\frac{H_2O}{0.33}$ cc.	Virus 2.7 cc. + $\frac{H_2O}{5}$ cc. + NaOH 0.5 per cent 0.33 cc.	Virus 2.7 cc. + alcohol 90 per cent 5 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 4.4 cc. + alcohol 90 per cent 3.3 cc. + $\frac{H_2O}{0.33}$ cc.	Virus 4.4 cc. + $\frac{H_2O}{3.3}$ cc. + NaOH 0.5 per cent 0.33 cc.	Virus 4.4 cc. + alcohol 90 per cent 3.3 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 6.0 cc. + alcohol 90 per cent 1.7 cc. + $\frac{H_2O}{0.33}$ cc.	Virus 6.0 cc. + $\frac{H_2O}{1.7}$ cc. + NaOH 0.5 per cent 0.33 cc.	Virus 6.0 cc. + alcohol 90 per cent 1.7 cc. + NaOH 0.5 per cent 0.33 cc.
1 min.......	+	+	−	+	+	−	+	+	+
2½ hrs.. ...	+	+		+	+		+	+	−**
6 "	+	+*		+	+		+	+	
24 "	+			+	+*		+	+*	
26 "	+			+*			+*		
30 "	−								

+ = virus active, − = virus killed.

* 6 hours was the longest time tested.   But 0.27 cc. of NaOH, 0.5 per cent, in a 7.7 cc. suspension did not kill the virus after 24 hours.

** Average time of 5 experiments.

small quantity used, that of 0.33 cc. to 8.03 cc. (or about 1 in 5000) of the medium.   But in the presence of the alkali, alcohol fails to coagulate the protein: the resultant mixture is clear.   Then the alcohol comes into direct contact with the unprotected virus and kills

it quickly; the 40 per cent and 60 per cent dilutions within 1 minute, and the 20 per cent, within an average time of $2\frac{1}{2}$ hours.[10]

We could make no comparison with other filter-passing viruses in respect to sensitiveness to alcohol under similar conditions. Hence we turned our attention to a definite living microorganism, the staphylococcus, for a relative study.

Comparison with Staphylococci.—In the following experiments cultures of *Staphylococcus aureus*, obtained from a patient with osteomyelitis, and grown on plain agar slants were substituted for the virus materials, and then subjected to alcohol action under similar conditions.

Protocol 4.—Suspensions of staphylococci were made in phosphate buffer at pH = 7.5. A proportional amount of this and of the stock 90 per cent alcohol was taken to make final concentrations of 60, 40, and 20 per cent of the latter, with the same quantities outlined in Table I for the virus-alcohol tests. This constituted 1 series, to check the next in which again 0.33 cc. of NaOH 0.5 per cent was added to the volume of 7.7 cc. of staphylococcus-alcohol suspensions. A tabulation of the results is given in Table II.

In respect to a suspension of staphylococci containing only micro-organisms which are not admixed with proteins as is the case with the virus, Table II shows that 60 per cent alcohol was capable of complete inhibition of growth within 1 minute. 40 per cent alcohol killed staphylococci after 6 minutes and 20 per cent after 3 days. But with this microorganism as well, the addition of the alkali increased somewhat its sensitiveness to the reagent: in 40 to 60 per cent alcohol the lethal action was too short to make a definite difference but in 20 per cent alcohol the killing time was shortened 2 days.

We may now make a comparison between the virus and staphylococci in regard to this phenomenon.

In 60 per cent alcohol the virus in its protein milieu remains alive for at least 26 hours—for a much longer time according to Abe and the workers of the British Commission. But freed from the protective coagulum, the virus is killed within 1 minute—so are staphylococci.

[10] In 4 instances the lethal point was $1\frac{1}{2}$ hours, 1 hour, or less, but in 1 instance which we cannot explain, the virus resisted 20 per cent alcohol plus NaOH for 7 hours. Stockman and Minett,[4] however, found that 25 per cent alcohol *per se* kills the virus after 3 days and 10 per cent after 20 days.

In 40 per cent alcohol, the virus in the guinea pig tissues or exudates is resistant for at least 26 hours, or even several days. In its free state, however, it is killed also within 1 minute. The growth of staphylococci is inhibited, on the other hand, after $5\frac{1}{2}$ to 6 minutes.

TABLE II.

Action of Alcohol on Staphylococci.

Time	Staphylococci + alcohol			Staphylococci + alcohol + NaOH 0.5 per cent 0.33 cc.		
	60 per cent	40 per cent	20 per cent alcohol	60 per cent	40 per cent	20 per cent alcohol
		No. of colonies	No. of colonies		No. of colonies	No. of colonies
min.						
1	—		Inf.	—	10	Inf.
$1\frac{1}{2}$		110	"			"
$3\frac{1}{4}$			"		5	"
$5\frac{1}{2}$			"		2	"
6		2	"		—	"
$6\frac{1}{2}$		—	"			"
60			"			"
hrs.						
$2\frac{1}{2}$			"			"
4 to 7			"			P. G.
8 to 22			P. G.			" "
23			" "			10
24			" "			—
days						
2			75			
3			8			
4			—			

— = no growth in subculture of 0.2 cc. Inf. = prodigious, normal growth as compared with controls. P. G. = profuse, coalesced growth making it impossible to count individual colonies.

Control cultures of the staphylococcus suspension showed the usual growth after 4 days.

In 20 per cent alcohol the virus in its natural state can remain viable for at least 3 days.[4] But when it is not adsorbed to coagula and there exposed directly to the action of this reagent it is killed in from less than 1 to 7 hours (in an exceptional case). Staphylococci,

on the other hand, are killed after 23 hours (with alkali) or after 3 days (without alkali).

In conclusion it may be stated that in testing viability of the virus admixed with proteins, one encounters complications due to the protective action of the protein coagula. When coagulation is prevented by taking advantage of the periodic phenomenon, and the virus is exposed *in toto* to the action of the chemical, it is then even more sensitive to destruction than a culture of a known microorganism, such as staphylococcus. The view-point, therefore, that the active agent of foot-and-mouth disease, by itself, is more resistant to alcohol than living bodies, such as bacteria, is incorrect.

Alcoholized Virus in Cultures.—It was believed that concentration of the virus could be effected by its adsorption to the coagulum which is formed by adding 60 per cent alcohol to the active agent in a protein milieu. By employing such "alcoholized" virus we also had in mind the possibility of removing ordinary, contaminating bacteria and, at the same time, the inhibiting substances which prevent growth *in vitro*. Results of experiments show that the virus prepared in this manner cannot be used for culture: for example, in buffered gelatin (described in the next paper on cultivation experiments), control virus survived at 34°C. for at least 9 days but no activity was noted with alcoholized virus on the 5th day of incubation.

Virucidal Chemicals.

It may be concluded from the observations already given that chemicals employed as antiseptics, which produce coagulation of proteins and lead to protection of virus adsorbed to the formed particles, may not be active as virucides. On the contrary, such substances which do not coagulate the proteins of the material containing the virus, thus placing one in more direct contact with the other, may act more powerfully as destructive agents. As the following will show we were justified in this belief.

The scope of this test was extended to include chemicals which are coagulating and those which are not, employing with both the same sample of virus materials and comparing their powers of destruction. Accordingly we chose as examples of the 1st group, such substances as bichloride of mercury and cresol, 2 samples. Later a

new preparation advanced as a certain antiseptic against the foot-and-mouth disease virus, chloronal,[11] was added. As specimens of the 2nd group of non-coagulating substances we selected antiformin and sodium hydrate.

In view of the fact that under actual field conditions coagulants are generally employed as virucides, most often cresol and bichloride of mercury, the results of the following experiments take on a greater significance.

The 1st experiment dealt with the virucidal properties of bichloride of mercury and 2 different samples of cresol, one labelled as such, and the other, as a cresol compound. Protocol 5 illustrates one such test:

Protocol 5.—Aspirated lymph virus was diluted in phosphate buffer at pH = 7.5. This form of virus was employed instead of fragments of infected tissue so as to favor any destructive action of the chemicals. To 2 cc. of 1:100 of the suspension of the active agent were added 2 cc. of 1:500 bichloride of mercury and to other respective lots, 2 cc. of 6 per cent cresol and *liquor cresolis compositus.* In the end the dilution of the virus was 1:200, of the bichloride 1:1000, and of the cresol preparations 3 per cent. After varying periods of contact, from 5 minutes to 6 hours (the longest time of testing), the mixtures were injected intradermally in guinea pigs. The results with all these substances were practically identical: Guinea pigs injected with material after 5 minutes to 2 hours contact showed primary vesicles in 24 hours and secondary lesions in 48 hours. Those animals injected with material after 4 to 6 hours contact exhibited primary lesions in 48 hours and secondary vesicles after 72 hours.

All the precautions mentioned in Protocol 3 were employed and great care was taken not to confuse chemical necroses with the lesions of experimental foot-and-mouth disease.

Hence bichloride of mercury, cresol, or compound cresol, all forming large coagula with virus materials, act as does alcohol. They do not destroy the virus quickly, at least, not after 6 hours, the longest period tested. On the other hand, in 2 other experiments chloronal in a 5 per cent solution, which substance forms much lesser and finer coagula, failed to destroy in 30 to 32 minutes but after 1 hour inactivated the virus.

[11] Trautwein, K., *Arch. wissensch. u. prakt. Tierheilk.*, 1925, lii, 254. According to this writer, chloronal contains 25 to 30 per cent chlorine, mainly in a dissociable state; but it is stated that it coagulates albumen.

The next step was to test the action of antiformin and sodium hydrate, substances which do not coagulate the proteins of the medium containing the virus.

Protocol 6 —Of antiformin, 1 per cent was employed; of sodium hydrate, ¼, ½, 1, and 2 per cent. The virus was diluted in phosphate buffer to 1·40 and, as a more severe test of the action of these reagents, consisted of ground infected pad tissue, unfiltered, and containing particles up to about 3 mm. in size. In other experiments in this series, aspirated lymph was also used as a source for virus; in still others the latter was diluted only 1:10. In all cases, however, the results were identical, and as follows:

Antiformin 1 per cent in contact with virus for 1, 2, 3½, 5, 15, and 60 minutes. All guinea pigs injected intradermally failed to show signs of the experimental disease.

Sodium hydrate ¼ per cent. 2 of 4 guinea pigs injected intradermally exhibited, in both cases, after 3 days, primary lesions of the experimental disease, with specimens in which the alkali and virus were in contact for 1 minute. The virus, however, after exposure for 3 minutes was inactive.

Sodium hydrate ½ per cent. 1 of 4 guinea pigs injected intradermally showed after 2 days primary vesicles and after 5 days, secondary lesions. Exposure of virus to the alkali 1 minute. After 3 minutes contact all the injected animals remained well.

Sodium hydrate 1 per cent in contact with the virus for 1 and 3 minutes. None of 4 guinea pigs injected were affected.

Sodium hydrate 2 per cent in contact with the virus (1:20 to 1·40 final dilution) for 1 to 3 minutes. Of 10 guinea pigs injected intradermally with the mixtures, all remained normal. Observations were also extended to cover a period of contact from 3 minutes to 1 hour. Again none of the latter injected animals was affected.

It is to be noted that variations in the technique outlined in Protocols 3 and 5 may lead to errors of interpretation. With antiformin and sodium hydrate, especially in higher concentrations, chemical necroses are apt to occur at the site of injection which may mask the primary lesions. Hence attention should be given to the appearance of secondary lesions. In addition, material from all suspected pads should be removed and suspensions thereof injected into a series of normal animals for control of the specificity of the reaction, and finally, whenever possible recovered animals should be tested for immunity.

These experiments demonstrate that chemicals such as antiformin and sodium hydrate, which do not coagulate the proteins of the medium containing the virus and therefore coming in direct contact with the active agent, were powerfully destructive to the incitant.

Antiformin in 1 per cent killed the virus within 1 minute.[12]   Sodium hydrate $\frac{1}{4}$ to $\frac{1}{2}$ per cent destroyed the active agent within 3 minutes but not in 1 minute.   At this concentration, however, the destructive effect was weak and irregular.   But in 1 to 2 per cent solutions, the inhibiting action occurred within 1 minute and was complete and regular.   It may be stated in favor of the completeness of this neutralizing effect, that the virus was penetrated and killed even when present in fragments of tissue as in ground infected pad tissues containing particles about 3 mm. long.

The question arose then as to whether soda could replace sodium hydrate in respect to the latter's powerful action.   But our experience with 5 per cent anhydrous sodium carbonate has shown that this could not be done.

The regular and energetic destruction of the virus by sodium hydrate in low concentrations led us to seek a practical application. Field conditions were imitated by admixing virus in bits of tissue with cow's urine, with manure, and with garden earth, and then exposing such mixed materials to the action of sodium hydrate.   An illustrative experiment, one of several, follows:

Protocol 7.—24 hours after injection, 4 infected pads were removed from guinea pigs and ground in 20 cc. of phosphate buffer at pH $= 7.6$. To this were added 20 cc. of aspirated lymph virus of a dilution of 1:10.   The material was unfiltered and contained a number of small particles of tissue.   2 cc. of this virus suspension were added to 2 cc. of urine from a normal cow.   The probable dilution of virus in this instance was 1:20.   2 cc. were also added to about 5 gm. of manure from normal cattle and to a similar amount of earth obtained from an area near the stalls.   In all instances the virus was thoroughly admixed with the substances to which it was added and the mixtures were allowed to stand at room temperature for 1 hour.

Then to the virus suspension itself, and to the urine, the manure, and the earth containing the virus, a quantity of sodium hydrate 4 per cent solution was added to make the final concentration of the latter 2 per cent.   Guinea pigs were injected intradermally with the mixtures exactly 1, 3, and 5 minutes after adding the alkali. Observations extended over 12 days showed that none of these animals became infected.

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[12] Stockman and Minett[4] have also found that the virus (in comparison with its great resistance to alcohol, ether, etc.) is easily destroyed, in "less than 1 hour," by antiformin 1 per cent.

At the end of an hour or after the completion of the test, portions of the virus suspension itself, and the urine, the manure, and the earth containing the virus, were injected intradermally in respective guinea pigs, which served as controls for activity of the different materials. In all animals typical primary lesions were observed 24 hours after injection and secondary vesicles 12 to 24 hours later.[13]

It appears, therefore, that sodium hydrate in a 2 per cent solution is capable of destroying, within 1 minute, not only the virus in its ordinary condition even when contained in fragments of tissue, but also such virus admixed with cattle urine, with manure, and with earth.

As a further application of the virucidal effect of this reagent to field conditions, the test was extended to include cattle as experimental animals. The results are shown in the following protocol.

Protocol 8.—The materials employed in this experiment were prepared in a manner exactly similar to that mentioned in Protocol 7. To portions of a suspension of virus, and virus mixed with cow's urine, with manure, and with earth,—in all cases thoroughly incorporated,—was added sodium hydrate in sufficient quantity so that the final concentration of the latter was 2 per cent (see Protocol 7). 1 minute later 9 cattle were inoculated by the method of scarification of the mucous membrane of the mouth and then rubbing in the substances quite briskly: 3 cattle were inoculated with the NaOH-virus material and 2 each with the remaining 3 mixtures, respectively.

1 hour later 3 normal cattle and 4 guinea pigs were inoculated, the former by the same scarification method employed in the test animals, with the suspension of the virus; and the latter intradermally, with the portions of the remainder of the 4 different virus mixtures. These animals served as controls for the activity of the virus and the latter admixed with urine, manure, or soil. In addition to these controls, a hog was injected intravenously and 2 convalescent heifers locally.

None of the cattle inoculated with the virus mixtures exposed to the action of sodium hydrate for 1 minute was affected, while all of the control animals except the convalescent heifers showed within 48 hours symptoms of experimental foot-and-mouth disease.

The 9 cattle employed in the test series—the 1st group—were inoculated, after 28 days, by the same scarification method, with active virus. All of them became ill with the typical experimental disease; thus demonstrating that the absence of effect from the first inoculation was not due to a natural resistance.

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[13] Care should be taken not to confuse chemical necroses with the true, typical lesions of experimental foot-and-mouth disease. (See Protocol 3, Paragraph 5.)

One may conclude from the foregoing that the destructive effect of 2 per cent sodium hydrate on the virus is complete and rapid—within 1 minute—as shown by tests not only on guinea pigs but on cattle as well.   This leads to the consideration of this reagent as an effective virucide in practice.[14]

Comparison of Action of These Reagents on Staphylococci.—We now turned our attention to the action of the different chemicals employed as virucides, upon definite living microorganisms such as staphylococci.   The object in such determinations was to note whether the

TABLE III.

Effect of Certain Chemicals on Staphylococcus aureus.

Time	Bichloride of mercury 1:1000	Cresol 3 per cent	Chloronal 5 per cent	Antiformin 1 per cent	NaOH 2 per cent	Control suspension of staphylococci
			No. of colonies			
min.						
1	1000	Inf.	4	14	200	Inf.
3	100	"	1	—	30	"
5	5	"	—	—	5	"
7	—	P. G.	—	—	—	"
10	—	1000	—	—	—	"
15	—	300	—	—	—	"
60	—	26	—	—	—	"
120	—	—	—	—	—	"

The indicating marks are similar to those used in Table II.

active agent of foot-and-mouth disease is by virtue of these reactions in a class by itself, or whether it can be correlated with living bacteria.

Protocol 9.—Suspensions of staphylococci were prepared as in the experiments cited in Protocol 4.   To these were added, respectively, bichloride of mercury in 1:1000, cresol and cresol compound, in 3 per cent, chloronal in 5 per cent, antiformin in 1 per cent, and sodium hydrate in 2 per cent, final concentrations.   After varying periods of time at room temperature, 0.1 cc. was subcultured on agar. The results are tabulated in Table III.

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[14] The limited time and space at our disposal prevented similar tests on cows with 1 per cent sodium hydrate.   But the results with guinea pigs demonstrate that this concentration of the chemical may be equally effective.

The results summarized in Table III reveal that pure cultures of staphylococci are killed by contact with bichloride of mercury 1:1000 after 5 minutes; by cresol 3 per cent, after 1 hour; by chloronal 5 per cent, after 3 minutes; by antiformin 1 per cent, after 1 minute; and by sodium hydrate 2 per cent, after 5 minutes.

The destructive action of these substances on pure staphyloccoci may now be compared with that on the virus as it is ordinarily employed in a protein medium. For greater ease of comparison the results of the latter experiments are summarized in Table IV.

TABLE IV.*

Effect of Certain Chemicals on the Virus (1:40).

Time	Bichloride of mercury 1:1000	Cresol 3 per cent	Chloronal 5 per cent	NaOH 2 per cent	Antiformin 1 per cent	Control of virus
min.						
1	+	+	+	−	−	+
3	+	+	+	−	−	+
5	+	+	+	−	−	+
15	+	+	+	−	−	+
20	+	+	+	−	−	+
30	+	+	+	−	−	+
32	+	+	+	−	−	+
hrs						
2	+	+	−	−	−	+
4	+	+	−	−	−	+
6	+	+	−	−	−	+

* The virus being either in the form of lymph or admixed with, adherent to, or incorporated in, small fragments of tissue. 6 hours was the longest period tested.

Table IV shows that the virus resisted the chemicals which caused protective coagulation of the proteins of its medium, but was killed within 1 minute by those which did not form a coagulum. On comparing the results given in Table III with those in Table IV, it will be noted that the active agent when not protected by coagula is still more sensitive to destruction by the same reagents, sodium hydrate and antiformin, than are staphylococci. Hence these tests confirm the prior conclusions with respect to the action of alcohol on the virus and on staphylococci. Furthermore, none of the effects of

these reagents on the virus can be interpreted as an indication of its inanimate character.

SUMMARY AND CONCLUSIONS.

The virus of foot-and-mouth disease exhibits a remarkable resistance to such bactericidal agents as the narcotic solvents (alcohol, ether, chloroform), or such antiseptics as phenol, bichloride of mercury, or cresol, as shown by tests made by others and ourselves. We have shown, however, that the resistance of the incitant to these chemicals is really masked. It is due to the fact that the reagents coagulate the proteins of the medium in which the virus is, as a rule, suspended. As a result the active agent is protected by the coagula which prevents direct contact with the chemicals. On the other hand, if advantage is taken of the periodic phenomenon attending such processes, and coagulation is prevented, the virus can then be brought under direct action of the antiseptics. Under these conditions, the incitant is more sensitive to destruction by the chemicals than is the living staphylococcus. As a corollary, the virus is destroyed as rapidly, or even more so, than are staphylococci by substances such as sodium hydrate (1 to 2 per cent solutions), or antiformin (1 per cent solution) which do not form coagula. We are therefore compelled to contradict the opinion that the extraordinary resistance to certain chemicals of the virus of foot-and-mouth disease, as it ordinarily occurs admixed with proteins, is an indication of its inanimate character.

The results of a large series of experiments lead to the conclusion that of a number of antiseptics employed the sodium hydrate in 1 to 2 per cent solutions is an effective virucide. It is capable of killing the virus within 1 minute as shown by tests on cattle and guinea pigs. Furthermore, its effectiveness is not diminished even when the virulent material is admixed with cattle's urine, with manure, or with garden soil. The experimental evidence and the cheapness suggest its use in field practice as a disinfectant.

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STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

IV. Cultivation Experiments.

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In the first paper[1] of this series we stated that the lesions induced by the foot-and-mouth disease virus are free from ordinary bacteria. But before undertaking an extensive study on cultivation, it was believed desirable to add to the routine morphological studies and to the use of some ordinary means of culture, a large variety of media under different atmospheric conditions, different temperatures, and with different sources of the active agent. In this way we thought that there might be revealed any constant, secondary or concomitant, microorganism.

Accordingly, a large number of experiments were made with the virus in the blood of actively infected guinea pigs, with filtered and unfiltered vesicular contents, and with filtered or unfiltered ground pad or foot tissues from guinea pigs or cattle. The materials were cultured in media at room temperature and at 32–37°C. The atmospheric conditions varied from aerobic to anaerobic and in either condition plus the addition of carbon dioxide (up to 25 per cent concentration). A variety of media, both fluid and solid, was used: ascitic fluid with 5 per cent guinea pig, defibrinated blood; ascitic fluid, calf tongue broth; 5 per cent sheep blood; 1 per cent dextrose calf tongue broth and these media with 2 per cent agar. Agar plates with rabbit or sheep blood were also employed.

No constant, visible microorganisms were detected in these artificial media, even after repeated subplants. Nor, in the absence of visible

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[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

growth, were we enabled to obtain a pathogenic culture sufficiently diluted to eliminate actual transfer of the virus. These preliminary tests showed, therefore, that the ordinary methods appeared to be unsuitable for the cultivation of the virus. Another conclusion was that only the filtered pad material and the blood were useful for cultivation: the unfiltered ground infected pad tissues or aspirated "lymph" were often admixed with the different bacteria normally present in the pads of guinea pigs or in the mouths of cattle, and the usual method of searing the surface was ineffective.

Our plan of study from this point was to make a deductive inquiry into the conditions which were best suited for the viability of the active agent *in vitro*. It was thought that this way of approach would yield more satisfactory results in our limited time than that of applying at once special methods of cultivation. But before this could be done, it was essential to investigate the findings of Frosch and Dahmen.[2]

A widespread interest was aroused in 1924 when these writers announced the cultivation by special methods, of minute bacillary bodies, *Loffleria nevermanii*, which they maintained were the inciting agents of foot-and-mouth disease. Their colonies, as well as the bodies themselves, were visible only by means of short wave-length, ultra-violet, photomicrography. Their method of culture consisted in centrifuging the virus materials (lymph) over a long period to concentrate the virus, and then washing the sediment in changes of salt solution to free the virus from inhibiting substances. The sediment was seeded on a solid, slope medium containing Martin broth, 3 per cent agar, to which was added, just before use, 20 to 50 per cent of horse or ox serum. The medium was adjusted to pH = 7.8. Growth occurred aerobically after 7 to 10 days at 33–34°C. Suspensions of these subvisible colonies, even to the 25th generation, induced in guinea pigs experimental foot-and-mouth disease, albeit at times the reactions were quite feeble.

In accordance with the statements of Frosch and Dahmen, prolonged centrifugation of the virus materials was a prerequisite for successful cultivation. We have already shown (*vide supra*) that this step in the technique does not concentrate the virus, nor can the so called inhibiting substances be thereby removed. Furthermore, by repeated tests the virus failed to survive in the Frosch and Dahmen medium after 3 days, although with the same specimen of virus and seeded at

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[2] Frosch, and Dahmen, H., *Arch. wissensch. u. prakt. Thierheilk.*, 1924, li, 99, 108; *Berl. tierärztl. Woch.*, 1924, xl, 185, 273, 341, 662.

Dahmen, H., *Berl. tierärztl. Woch.*, 1925, xli, 733.

the same time, it remained viable for 7 days in buffered 10 per cent gelatin at pH = 7.6 and for 9 days in buffered phosphate[3] at pH = 7.6. We will show later that broth is an unsuitable medium; this applies with equal force to the serum. In addition, the hydrogen ion concentration and the aerobic conditions which are employed in this method were also found unsatisfactory.

We could not confirm the conclusions of Frosch and Dahmen, nor were we favored by finding therein, on analysis, any clew which might lead to a lengthening of the life of the virus under artificial conditions. In respect to our lack of confirmation of Frosch and Dahmen's results we are in agreement with the German Commission directed by Dr. Gins,[4] who also found that in Martin broth serum agar the virus dies in 3 days. We are also in accord with the work of the British Commission.[5-7] These investigators have shown that the so called colonies and their constituents could be imitated by materials other than the virus of foot-and-mouth disease.

In view of the importance generally ascribed to the hydrogen ion concentration of media in affecting growth or viability of microorganisms, our earlier studies were directed to the optimum range required for the virus.

Optimum Hydrogen Ion Concentration.—As the following illustrative protocol shows, variation in the hydrogen ion concentration of the media containing the virus affected its viability.

Protocol 1.—The same specimen of virus, 24 hour old aspirated lymph in phosphate buffer and filtered through a Berkefeld candle, was employed in all these tests. ½ cc. was inoculated into each 10 cc. of the medium to be stated.

(a) Buffered 10 per cent gelatin at pH = 6.9 after 5 days' incubation at 34°C. 2 guinea pigs injected intradermally. Both negative.

Buffered 10 per cent gelatin at pH = 7.5 after 5 and 7 days' incubation at 34°C. 4 guinea pigs injected, 2 each respectively with the 5 and 7 day incubated materials. All positive.

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[3] For its preparation Papers I and II should be consulted.

[4] Gins, H. A., *Berl. tierärztl. Woch.*, 1924, xl, 661; abstracted in *J. Comp. Path. and Therap.*, 1924, xxxvii, 302.

[5] First Progress Report of the Foot-and-Mouth Disease Research Committee, Ministry of Agriculture and Fisheries, London, 1925.

[6] Arkwright, J. A., Burbury, M., Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[7] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

(*b*) Buffered agar ¼ per cent at pH = 8 after 3 days' incubation.   2 guinea pigs injected intradermally.   Both negative.

Buffered agar ¼ per cent at pH = 7.6 after 3, 5, 7, and 9 days' incubation. 8 guinea pigs injected, 2 each respectively with a portion of each specimen.   All positive.

These instances do not show the extreme effect of variations of hydrogen ion concentration on the preservation of the virus: in 1 case in 2 per cent dextrose, 5 per cent sheep blood, calf tongue extract medium the latter survived 25 days longer at room temperature at pH = 7.4 than at pH = 6.   In general, a large experience demonstrated that the optimum hydrogen ion concentration best suited for the viability of the active agent *in vitro* is at pH = 7.5 to 7.6.   Variations above or below this narrow range exhibit an unfavorable influence.   We are thus in agreement with the findings of Stockman and Minett[7] and of Bedson and Maitland[6] of the British Commission. Conversely, it became apparent from a number of tests which need not be quoted here, that the phosphate buffer solution at pH = 7.5 or 7.6 could be employed to greater advantage as a base for the preparation of a wide variety of media used in tests of their preserving action, than media not prepared on this base.

Temperature.—The active agent is very sensitive to higher temperatures.   We have already stated that the virus in guinea pig blood survives after 24, but not 48 hours at 37°C.   The following protocol exemplifies the advantageous effect of lower temperatures.

Protocol 2.—Virus material consisted of a 1:100 dilution of aspirated lymph in phosphate buffer, adjusted to pH = 7.6 and filtered through a Berkefeld candle. One portion was kept at 37°C., another at 32–34°C., and a third at 18–20°C. (room temperature).   After varying intervals of time, the materials were injected intradermally in guinea pigs.   The virus retained at 37°C. survived for 3 days but not for 5; at 32–34°C. for 9 days but not for 11; and at 18–20°C. for at least 25 days.   In addition, in this medium plus 50 per cent glycerol the virus remained alive indefinitely in the ice box (2–5°C.).

In another test with a more complex medium, the dextrose, sheep blood, calf tongue extract mentioned kept under anaerobic conditions, the virus survived herein for 6 days at 37°C., and for 25 but not for 32 days at 18–20°C.

From these and other observations to be described later it appears that the virus of foot-and-mouth disease like that of mosaic disease[8]

[8] Johnson, J., *Phytopathology*, 1921, xi, 446; 1922, xii, 438.

and other filter passers was sensitive to higher but was quite resistant to lower temperatures. At 37°C. we were unable to keep the active agent alive for more than 6 days. At 33–34°C. viability was maintained for 10 days; at 18–20°C. for from 2 weeks to over 2 months (in an instance to be described later), and at ice box temperature, indefinitely. These observations concur in the main with those of the workers of the British Commission.

Oxygen Tension.—The importance of the question whether the life of the virus is favored by aerobic or anaerobic conditions was appreciated. Hence a number of repeated tests in a variety of media was made, some of which are exemplified in the following:

Protocol 3.—24 hour old virus in the form of aspirated lymph 1 to 20 cc. phosphate buffer at pH = 7.6 was filtered through Berkefeld candles. Of the filtrate 1 cc. was added to each 10 cc. of the medium which was always placed in Petri dishes so as to give it as much surface exposure as possible either, to the aerobic or to the anaerobic atmosphere.

Medium of 10 per cent gelatin prepared by Loeb's method (to be described later). After 5 days' incubation guinea pigs were injected respectively with aerobic and anaerobic materials. Those injected with aerobic subplant showed after 48 to 72 hours mild primary lesions and 24 to 48 hours later similar secondary vesicles. The others, injected with anaerobic material, exhibited within 24 hours severe primary vesicles and 24 hours later many secondary lesions. After 7 days' incubation, aerobic subplants were inactive; the anaerobic could induce the typical experimental disease after 48 hours. After 10 days' incubation, anaerobic material still showed activity in 1 of 3 guinea pigs.

The same general results were obtained when other media were employed.

These experiments indicate that under strict anaerobic conditions the virus retains its viability at least twice as long as under aerobic conditions. The investigators of the British Commission (Stockman and Minett[7]) report, however, that the state of air tension is of no importance from the view-point of survival. The discrepancy in results may be ascribed to a difference of technique. We have employed not only Petri dishes for the tests but also the Boëz apparatus for producing anaerobic conditions.[9] This mechanism employing vacuum, consists of a central post catalyzing station for the combustion of oxygen. It is leak-proof and establishes a perfect and perma-

[9] Boëz, L., *Compt. rend. Soc. biol.*, 1925, xciii, 1663, 1666; 1926, xcv, 708; *J. Bacteriol.*, 1927, xiii, 227.

nent anaerobic atmosphere within a few minutes. None of the methods employed by the British, such as capping tubes, or using a vacuum (Geryk) pump, or exhausting air with or without a hydrogen atmosphere, or boiling, then quickly cooling the medium, and later adding vaseline is comparable to the Boëz apparatus in promptness and effectiveness.[10] Indeed, it may be questioned whether a strict anaerobic condition was ever secured by the British methods.

Influence of Different Media.—The object of the following experiments was to study the effect of different media upon the duration of life of the virus. We began with a series of substances which comprised the Frosch and Dahmen medium, namely broth, serum, agar, and also Witte and Martin peptones.

A. Broth.—We have already stated that the virus died within 3 days in Martin's broth. But no greater success was obtained after repeated trials, with plain broth, buffered broth, tryptic broth, broth prepared with calf tongue instead of beef infusion, and beef infusion itself. In view of the fact that in other media, such as simple buffer phosphate or 10 per cent gelatin, the virus survived much longer under the same conditions of temperature (34°C.) and in parallel experiments, it is apparent that broth is noxious to the active agent. Furthermore, when materials, for example, 10 per cent gelatin, which in themselves were capable of maintaining the life of the virus for a considerable period, were added to broth, not only was the latter unimproved but the favorable action of the intended adjuvant was also lost. As, for example, in 10 per cent gelatin broth the virus did not survive after 3 days, but in the gelatin itself it remained viable after 9 days.

Hence broth including the Martin variety may be considered not only as an unfavorable medium but, moreover, as a rapidly destructive agent to the virus.

The question naturally arose as to what part the peptones of the broth played in this regard. They were therefore put to test.

[10] It was determined, for example, that *Bacterium pneumosintes*, a strict anaerobe, failed to grow by methods depending on the mechanical extraction of air *per se*. In regard to the method of the vaseline seal, the cooling of the medium permits a prompt reabsorption of air (see Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51).

B. Peptones.—2 samples of peptone were available for study: Martin's and Witte's. The following protocol, presenting 1 of many experiments, exemplifies their action.

Protocol 4.—Aspirated lymph, 24 hour old virus was diluted 1:50 in phosphate buffer at pH = 7.5. This was filtered through a Berkefeld candle and 1 cc. of the filtrate was added to 10 cc. of each of the media to be stated. All were incubated at 34°C.

Medium A comprised phosphate buffer at pH = 7.5.

Medium B, the same plus 1 per cent Martin's peptone, and·

Medium C, the phosphate buffer plus 1 per cent Witte's peptone.

After 3 days' incubation portions of each were inoculated intradermally into each of 2 guinea pigs, respectively. Within 48 hours all animals showed the typical primary and secondary lesions.

After 5 days' incubation, Medium A could induce primary lesions within 24 hours; Media B and C, much weaker reactions after 48 to 72 hours.

After 7 and 9 days' incubation, Medium A was still active in both of the 2 guinea pigs; Media B and C, inactive.

In addition to this experiment, another was made in which 1 per cent Martin's or 1 per cent Witte's peptone was added to buffered agar or buffered washed 10 per cent gelatin, thus making 4 distinct combinations.

The virus remained alive in the agar (0.25 per cent) for 9 days but in the agar with Witte's peptone for only 5 days and in the agar with Martin's peptone for only 3 days. The virus survived in the 10 per cent washed gelatin for 7 days but in the gelatin with Martin's or Witte's peptone for 5 days. In the latter instances only 1 of 2 guinea pigs showed a feeble reaction after injection.

This experiment shows that Martin's and Witte's peptones are not only unfavorable for the preservation or viability of the virus but that they also tend to exert a harmful effect when added to media which by themselves are favorable to preservation. In this action a counterpart is found in broth. Peptone is one of its constituents which is noxious. Since, however, the period of survival in peptone is somewhat longer than in broth, it appears then that besides it another harmful agent may be present. We have not as yet been able to identify this substance.

C. Serum.—What has been stated for the unfavorable effects of broth or peptone on the survival of the virus applied with equal weight to fresh animal serum and also to human ascitic fluid. For example, the virus remained alive in buffered 10 per cent washed gelatin for 7 days at 34°C., but in this medium with 50 per cent fresh

sheep serum, the same sample of virus was inactive after 5 days of incubation. Repeated tests have shown that fresh sheep or horse serum, or ascitic fluid, added to such substances as agar or gelatin, which by themselves are favorable to the preservation of the virus, exert a virucidal effect. Serum or ascitic fluid also does not counteract the injurious action of broth. Hence these materials are valueless as adjuvants in artificial cultures.

D. *Agar.*—3 per cent agar is the final ingredient of the Frosch and Dahmen medium. We have found that 2 to 3 per cent agar has an unfavorable influence on the virus for the latter does not survive in this medium longer than 3 days while in other media under the same conditions it survives for a much longer period. However, we extended our studies on the influence on lower concentrations of agar with the hope that perhaps a semifluid, rather than a solid medium might be favorable. The next protocol gives the results obtained.

Protocol 5.—The virus employed in these tests consisted of aspirated lymph diluted 1:30 in phosphate buffer at pH = 7.6 and filtered. 1 cc. of the filtrate was added to 10 cc. of each of the following specimens of agar. The agar was thoroughly washed in distilled water, buffered by the phosphate buffer to pH = 7.6, and prepared in concentrations of 0.1 per cent, 0.25 per cent, and 0.5 per cent. After 5, 7, 9, and 11 days of incubation at 34°C., a portion of each of the inoculated agars was injected intradermally in at least 2 guinea pigs, respectively, for each test. The virus survived in the 0.1 per cent and 0.5 per cent agar for 7 but not for 9 days, but in the 0.25 per cent agar for 9 but not for 11 days.

It is of interest to note that the optimum was at 0.25 per cent of the concentrations of buffered, washed agar employed. In this simple medium the virus survived for 9 days. But as we have already stated, the addition of 1 per cent, Witte's or Martin's peptone, 50 per cent beef maceration, 50 per cent fresh sheep serum, or 50 per cent ascitic fluid, shortened the viability period to 5 days or less. In addition further tests were made with 1 per cent lecithin, 1 per cent dextrose, 4.6 per cent glycerophosphate, and 2 per cent sodium citrate. When these latter substances are added, respectively, to the 0.25 per cent agar, the life of the virus is again shortened to less than 5 days.

It appears from the foregoing, furthermore, that the survival of the active agent depends on the simplicity of the medium in which it is contained—a principle amply confirmed by later tests.

The next step was to test the survival of the virus in 0.25 per cent agar at a lower temperature, namely at 18–20°C.

Protocol 6.—The virus consisted of a filtrate of infected pads, removed 24 hours after injection, ground in phosphate buffer at pH = 7.5. The filtrate, the final dilution of which was 1:100, was injected intradermally in 2 guinea pigs which promptly reacted with the typical primary and secondary vesicles. (Although not mentioned in the prior protocols, this routine procedure was always carried out.) Thoroughly washed agar 0.25 per cent and buffered with the phosphate buffer to pH = 7.5 comprised the medium. To each 10 cc., 1 cc. of the filtrate was added, and the mixtures were kept at 18–20°C. At intervals of 7, 12, 18, 24, 35, 52, and 76 days, portions of this semisolid medium were injected intradermally in at least 2 guinea pigs. It was found that the specimens incubated for from 7 to 52 days were active in the animals but that of the 72nd day, inactive.

Subplants were made from the 1st series of inoculated media after 7 days' incubation into fresh agar. This 2nd transfer also showed activity. The 2nd transfer after 6 days' incubation was subplanted to fresh similar medium. The 3rd was still active. The 4th subplant, however, failed to affect guinea pigs. This test of subplanting the virus was repeated after different periods of incubation and the results were always the same: activity was shown in the first 3 transfers but not so in the 4th—with the proviso that the 3rd transfer was within 52 days after the original seeding of the medium.

In these tests we also encountered a minute bacilloid microorganism which was filter-passing through Berkefeld "V" filters in the 1st but not in later generations. It also grew in peptone and beef maceration semifluid agar. In transferring this microorganism, an admixture of it with some of the virus medium was employed, for it was impossible to obtain a pure culture free from virus. Material containing this microbe was also infectious in the 3rd subplant but not in the 4th.

From the foregoing one may conclude that 0.25 per cent agar is a favorable medium for the preservation of the virus, at least, it is not toxic to it, for it survived in this medium for 52 days at 18–20°C. In view of our later experiences with gelatin which acted still more favorably than agar, it appears that the beneficial effect of the latter is due to its physical property of providing a semisolid structure to the medium.

This experiment also demonstrates that activity in 3 successive subplants was obtained not only with the virus but also with a microorganism for which we have sufficient evidence to indicate that it is merely concurrent with the particular sample of virus studied, and cannot be identified as the active agent itself. But the 4th subplant

in both these instances failed. Since the 4th transfer is the critical one (as explained in Paper I of this series[1]), for in this the virus is diluted beyond its original limit of activity, the infectivity of both the virus and the microorganism—which was maintained not in pure culture but admixed with the virus in the medium—can be interpreted as an expression of preservation but not of multiplication.

E. Gelatin.—The advantage of 10 per cent gelatin, which forms a semisolid medium, over other substances as a preservative of the virus soon became apparent and we were thus led to an extensive study of this material.[11]

It is well known that gelatin contains, as impurities, metals such as arsenic and copper and a considerable concentration of acids. A comparative test was made first with the same sample of virus added to the ordinary Poulenc 10 per cent gelatin and this substance thoroughly washed in distilled water. Both media were buffered at pH = 7.6. The active agent survived in the unwashed gelatin for about 3 days but in the washed, 7 days.

Our next effort concerned the probability of prolonging the preserving action of the washed gelatin by adding to it a number of organic or protein substances:

Protocol 7.—To different lots of 10 per cent washed gelatin prepared on the phosphate buffer base at pH = 7.6 were added, respectively, calf tongue broth, an equal part; Martin's, tryptic, or Witte's peptones, in 1 per cent concentrations; aspargine 0.5 per cent; dextrose 2 per cent, or fresh sheep serum, an equal part. As a control medium, buffered washed agar 0.25 per cent was employed. To each of these substances was added 24 hour old aspirated lymph virus diluted 1:30 in the phosphate buffer, pH = 7.5, and filtered. After varying periods of incubation at 34°C., portions from each were removed and injected intradermally in guinea pigs. The virus survived in the agar for 9 days, in the washed gelatin for at least 7 days, but in the mixtures of organic and protein substances with the washed gelatin, not longer than 3 or 5 days.

Thus, as is the case with agar, the addition of protein or other organic substances has a distinctly deleterious effect: the virus

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[11] The protein gelatin also is favored by its simplicity of composition. Its minimal molecular weight is estimated at 10,300 while that of hemoglobin at 50,000. For comparison, furthermore, the minimal molecular weight of egg albumen is stated as 33,800; of fibrin, 42,000; of serum globulin, 81,000; and of casein, 192,000 (see Cohn, E. J., Hendry, J. L., and Prentiss, A. M., *J. Biol. Chem.*, 1925, lxiii, 721).

requires for its survival only the simplest material. From this point, studies were therefore made on the effect of the gelatin alone.

We then employed a more thoroughly purified gelatin. Accordingly, we prepared the substance after the manner of Loeb for making isoelectric gelatin.[12] The method of preparation was as follows:

50 gm. of Poulenc powdered gelatin were added to 3 liters of M/128 acetic acid at 10°C. and stirred frequently. After 30 minutes the supernatant liquid was decanted, and fresh M/128 acetic acid at 10°C. (or in the cold room) was added to equal the original volume. The mass was frequently stirred and after ½ hour the acid was again decanted and replaced by an equal volume of distilled water at 5°C. The gelatin was well stirred and then filtered by suction in a Büchner funnel. It was then washed in the funnel 5 times with 5 liters of cold water. After all the water was drained off, the gelatin was removed and suspended in the buffer phosphate to a concentration of 10 per cent. It was then heated in the autoclave for sterilization on 3 successive days for 1 hour at 100°C. and the pH was adjusted to 7.6 between the heatings and after the final sterilization.

We then compared the effect of this buffered Loeb gelatin with buffered 0.25 per cent agar and found that the former permitted the survival of the virus for a much longer time than did the agar. For example in 1 experiment, the same sample of virus remained alive in the gelatin for 11 days at 34°C., but in the agar for less than 5 days. Hence the Loeb gelatin was not only superior to the same material washed by ordinary methods, but it also surpassed agar.

We then simplified the Loeb gelatin still further by omitting the buffer phosphate solution and adjusting the material to pH = 7.5 or 7.6 merely with potassium hydrate. Comparative tests revealed that the "adjusted" gelatin maintained the virus for longer periods than the buffered, at either 34°C. or 18–20°C., and under either aerobic or anaerobic conditions.

In the adjusted Loeb gelatin, the virus was kept alive for a longer period than in any of the other media tested. This period was longer than 69, but shorter than 100 days when the material was kept at room temperature (18–20°C.). In a parallel experiment, the active agent remained viable in 0.25 per cent agar for 45 days.

In this medium 3 successive subplants showed activity, as in the

[12] Loeb, J., Proteins and the theory of colloidal behavior, New York, 2nd edition, 1924, 41.

case with the agar. But we regard this fact as merely indicative of preservation of the virus rather than of its multiplication. For the 4th subplants—the critical ones, the ones in which the virus is diluted beyond its original limit of activity—were uniformly negative.[13]

At this point, when we had developed the optimum medium for the preservation of virus, namely, the adjusted Loeb gelatin, the limitation of time compelled us to discontinue the tests.

While these experiments were in progress we attempted the cultivation of the active agent in a variety of other special media and by different methods. We report these here for their general interest.

Miscellaneous Cultivation Tests.—Smith-Noguchi medium.[10] In this medium, the virus was incubated at 32–34°C., and subplants were made after 3 days. Material of the 1st subplant induced the typical experimental disease in all of 3 guinea pigs injected but that of the 2nd and of the 3rd successive transfers was inactive. In similar medium without the vaseline seal and incubated at 32°C. in an atmosphere of 10 per cent carbon dioxide combined with complete anaerobiosis identical results were obtained. In the Smith-Noguchi medium, therefore, the virus was preserved for about 3 days, or in the 1st subplant. But no evidence was obtained to indicate that the active agent could reproduce itself therein.

Tissue Cultures.—In tissue cultures made after the manner of Borrel, somewhat better results were obtained. These cultures were made with chicken plasma, chicken or guinea pig embryonic juice, and with fragments of embryonic epithelium. It was found possible to maintain the life of the virus through the 2nd subplant or for 4 days. The 3rd and subsequent successive transfers, however, were uniformly inactive. This method, therefore, also failed to favor multiplication of the virus. In the later transplants we frequently encountered secondary organisms so that the results of tissue cultures are inconclusive.

Adjusted Loeb 10 Per Cent Gelatin.—In this medium still better results were obtained. At 32–34°C. the virus was found in 3 successive subplants—in other words over a period of 9 days from the time of seeding. But later transfers were negative. Hence no multiplica-

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[13] It is of interest that the 3rd subplants were maintained at a temperature of 34°C., as well as at 18–20°C. In either case the medium preserved the virus.

tion was noted here as well. Efforts were made to employ this medium in a 10 per cent carbon dioxide atmosphere but this method proved less favorable—the 1st subplant was the only one positive. The addition of a fragment of fresh rabbit kidney to this gelatin showed no better results, but this is additional proof that the requirements of the virus for life postulate the simplest structure of the medium. Whether our experiences with this medium can be interpreted to indicate that the conditions of preservation are distinct from those of multiplication or whether the medium can be still further improved to the point of making it a basic material for successful artificial cultivation, remains at present an open question.[14]

General Considerations on the Particulate and Living Character of the Virus.

We propose to consider the questions which have often been discussed as to whether the filter-passing virus of foot-and-mouth disease is an inanimate, chemical material, or a multiplying living body; or whether it is fluid, as postulated by the theory of *contagium vivum fluidum* (Beijerinck), or particulate. For a justification of experiments on artificial cultivation rests upon the assumption that the virus may be particulate and living.

With reference to the theory of *contagium vivum fluidum* it appears that the definition of this term is not in accord with the modern concept of the particulate structure of matter. The burden of proof that fluids, in general, may not be corpuscular rests then upon the

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[14] We intend that this be the last paper of this series. The forthcoming report of the Commission to be published by the United States Bureau of Animal Industry should be consulted for additional miscellaneous studies by the writers and by Drs. Schoening and Traum. These include: Experiments on respiration of the virus, which were discontinued for want of a suitable control, and on intracutaneous reactions with concentrated virus. The latter tests revealed no practical procedure for skin diagnosis of foot-and-mouth disease, although it was noted that there was a certain cuticular sensitiveness of guinea pigs with the active experimental disease to the living virus. In addition it was found that earthworms could not be implicated as carriers and, contrary to the opinion of some observers, the virus is not fragile outside the animal body for it survived in earth for at least 25 days. In this report will also be found the observations of the Commission as a whole on epidemiology, immunity, and the clinic of foot-and-mouth disease, and some studies on the virus of vesicular stomatitis.

partisans of this theory. Apart from this we have presented what we believe as sufficient evidence that the active agent of foot-and-mouth disease is particulate. For by repeated and controlled filtration tests we have not only delimited measurement of individual active masses as relatively between 20 to 100 mμ, but also found that this relative size is constant.[15] The invariability of the limits of size contradicts, therefore, the notion that the incitant may be a "solute" varying in size in different "solvents." The electric charge carried by these particles, has, moreover, been determined by cataphoresis which can effect their separation from the protein substances in the medium containing virus.[16]

With regard to the question of the living character of the virus. its activity in extraordinarily high dilutions, its non-centrifugability, its remarkable resistance to such bactericidal agents as the narcotic solvents (alcohol, ether, chloroform) or such antiseptics as phenol, cresol, bichloride of mercury, etc., tend to support the idea that it is inanimate, or a chemical substance. We believe, nevertheless, that nothing has as yet been brought forward in these respects to prove conclusively that the active agent is inanimate. The factors just mentioned may represent reactions consequent upon its minuteness of size or its electropositive charge or both. It should be remembered that minute particles are subject to different physical laws than larger structures (Vlès[16]). For example, internal pressure, surface tension, and charge exert profound effects. However this may be, if each of the factors mentioned as opposed to the notion of a living nature of the incitant is analyzed, as will be done immediately, it will be noted that none is incompatible with this idea.

We have found that active materials are still infective in a dilution of 1:10,000,000.[1] This can be interpreted as merely indicating its minute size—yet sufficiently large in volume to contain from several hundred to several thousand protein molecules.[15] On the other hand, the action of the virus stops at a definite dilution and is directly proportional in respect to the length of incubation period and severity of symptoms upon its concentration. We have not encountered any effect similar to that noted in certain enzymatic actions, of nullifying antibodies in low dilutions.

[15] Olitsky, P. K., and Boëz, L., J. Exp. Med., 1927, xlv, 685.

[16] Vlès, F., Rev. scient., 1921, lix, 294.

As for the inability to depose the virus in a fluid medium by centrifugation, we have already explained[1] that this method is not useful for sedimenting minute particles: whirling and convection currents and other forces hinder the deposition. This property is therefore also a consequence of its size and does not indicate, by itself, a fluid material.

The resistance to chemical reagents is, as we have shown, really illusory.[17] It is due to the fact that these reagents coagulate the proteins of the medium in which the virus is as a rule suspended. As a result the virus is protected by the coagulum and direct contact with the reagent becomes impossible. If coagulation is prevented, which can be done, the virus is then acted upon directly by the antiseptics. The incitant is then more rapidly destroyed by the chemicals than are living bodies such as staphylococci.

Finally, among analogies to living microorganisms, may be mentioned the epidemiological factors, the existence of at least 2 distinct types of virus, the more or less solid immunity which is induced, and a period of incubation which can at times last 12 days. Studies of these conditions will be described in detail in the Commission's complete report. On the other hand, if the virus is a living body, it appears to be of an order wholly different from that of known cultivable microorganisms. For, aside from its electropositive charge, deductive study of its requirements for artificial life indicates that the metabolism of the incitant is of a very simple kind and is quite limited to definite and inflexible conditions.

We may therefore conclude that nothing has as yet been presented to prove that the virus of foot-and-mouth disease is of a fluid character or inanimate nature.[18]

SUMMARY AND CONCLUSIONS.

No multiplication of the virus *in vitro* was observed. We have found, however, that the optimum conditions necessary for the preservation of the virus in artificial media are as follows:

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[17] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815.

[18] This problem is still being studied by one of the writers (O.) but with the virus of vesicular stomatitis of horses. This virus is similar in clinical manifestations and in many biological reactions, to be reported later, to those of foot-and-mouth disease. In a recent collaboration with Dr. F. L. Gates of The Rockefeller In-

The hydrogen ion concentration of the medium should be 7.5 to 7.6, not only at the beginning, but, and more important, at the conclusion of the period of observation. A strict anaerobic atmosphere is also favorable, as is a temperature below 37°C. A semisolid structure of the medium appears to be advantageous and this can be effected by the use of $\frac{1}{4}$th per cent agar or 10 per cent gelatin. Of the two, the gelatin is more desirable, and of the latter the most effective material is gelatin from which the impurities have been most thoroughly removed, namely the gelatin employed by Loeb for his isoelectric determinations. This material is best adjusted to the proper hydrogen ion concentration (7.5–7.6) with potassium hydroxide and not with buffer phosphate.

Gelatin is the simplest of protein media available and its employment is in keeping with the principle we have found that the requirements for life of the virus of foot-and-mouth disease are of the simplest. The addition of organic or complex protein substances, such as dextrose, broth, serum, lipoids, etc., to a simple basic medium interferes with the effectiveness of the latter. It is thus not surprising that we were unable to confirm the cultural results of Frosch and Dahmen for neither their medium nor its components or their method satisfies the essential conditions necessary to maintain artificially the life of the virus.

Furthermore, from the standpoint of technique it was found necessary when comparing 2 or more media for their value in preserving the incitant, to employ all of them in a parallel experiment with the same sample of virus, for the factors of potency of the active agent, contamination, and changes in hydrogen ion concentration, if variable, may give rise to faulty interpretations. Moreover, activity in 3 successive subplants may be regarded as mere preservation but not multiplication of the virus.

Finally, we discuss the status of the virus from the point of view of its fluid or particulate, and its animate or inanimate, characters.

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stitute it was found that the virus of vesicular stomatitis is destroyed by the same limits of energy and wave-length of ultra-violet light which can kill staphylococci. "Since the absorption of specific energies is one index of chemical character, and in this instance results in similar effects, these parallel reactions are indirect but suggestive evidence that the substance of the virus is similar in character and chemical constitution to bacterial protoplasm" (Olitsky, P. K., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 431).

OXYGEN POISONING IN MAMMALS.

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PLATES 27 AND 28.

INTRODUCTION.

The increasing use of oxygen in clinical medicine would seem to
make worth while a study of the toxic effects which may result from
its prolonged inhalation in high concentrations. The observation
that oxygen inhalation may lead to pathological states is not a new
one.   And yet it has seemed important to us, because of our own con-
stant use of oxygen inhalation as a therapeutic measure, to investigate
this problem further, giving particular attention to the nature of the
injury and the mechanism of death.   The fact that many of the ex-
periments done in the past were performed on animals housed in small
containers with inadequate ventilation made it appear especially
desirable to repeat experiments under conditions in which CO_2
removal, temperature, humidity and air motion were controlled, and
to rule out definitely the possibility of death resulting from respiratory
infections so prone to occur in caged animals and in rabbits in particu-
lar.   It seemed not improbable that some of the pulmonary lesions
described in the literature might be the result of respiratory infections
perhaps resulting from a lowered resistance of lung tissue when ex-
posed to an abnormal mixture of atmospheric gases.

Lavoisier is said to have remarked the poisonous effects of oxygen inhalation
shortly after the discovery of this gas in the atmosphere. Regnault and Reiset
(2) in 1849 demonstrated that the respiration of different species of animals in an
atmosphere containing two or three times the normal concentration of oxygen
remains unchanged. They found no alteration in the amount of oxygen con-
sumed, in the respiratory quotient, or in the general behavior of the exposed ani-
mals. No further investigation in this field appears to have been made until the

classical experiments of Paul Bert (3) established the fact that oxygen at high
tensions is a powerful poison.   Bert showed that sparrows exposed to atmospheric
air at 15 to 20 atmospheres pressure developed convulsions and died.   If pure
oxygen were used in the compression chamber only one-fifth as much pressure was
needed to produce the same effects.   On the other hand, if oxygen-poor air were
used in the compression chamber the sparrows survived until overcome by the
accumulated CO_2.   Bert concluded that the toxicity of highly compressed air
was due to the high tension of the contained oxygen, which he believed exerted a
direct toxic effect upon the central nervous system.   He was unable to reproduce
the symptoms by the injection into a normal dog of a large amount of blood taken
from a dog in oxygen convulsions.   From this he argued that the status epilepticus
which he described was not the result of a circulating poison secondarily produced
by the oxygen.   He found that high oxygen tensions had a similar effect on other
laboratory animals and was also injurious to insects, arachnids, myriapods,
molluscs, earthworms and germinating seeds.   It inhibited the putrefaction of
meat and delayed the souring of milk.

20 years later the problem was taken up by Lorrain Smith (4), who was the first
to test out the effect of prolonged exposures to moderately high oxygen tensions.
He found that mice suffered no ill effect from exposure for 8 days to 41.6 per cent
of an atmosphere of oxygen, but that with 70 to 80 per cent of an atmosphere some
mice died in 4 days of "congestion and consolidation" of the lungs, while others
survived.   At 1.14 to 1.50 atmospheres of oxygen all the mice died of consolidation
of the lungs in 40 to 70 hours.   At 1.66 to 1.89 atmospheres of oxygen mice, guinea
pigs and larks died at 7 to 27 hours, the lung changes being similar.   At 3.55 to
3.57 atmospheres of oxygen mice died in 5 hours.   Mice which had begun to show
dyspnea on exposure to this tension of oxygen died at once on being taken out of
the chamber.   Two larks exposed to 3.017 atmospheres of oxygen developed con-
vulsions in 10 minutes.   They were taken out of the chamber after 2 hours but
both died.   There was nothing noteworthy in their postmortem appearances.
Lorrain Smith showed that by a previous short exposure to a high oxygen tension
or by raising the oxygen tension very slowly the exposure could be carried dis-
tinctly beyond the point which usually produces convulsions.   He also aimed to
show that the toxic effect of oxygen is related to its tension in the inspired air and
not its quantity in the blood.   In a chamber containing 0.4 per cent carbon monox-
ide as well as 3 atmospheres of oxygen larks developed convulsions as usual,
although the arterial blood at the end of the experiment was only 38 per cent
saturated with oxygen.

In brief, Lorrain Smith demonstrated that moderately high tensions of oxygen
produce an inflammation of the lungs, while very high tensions have an irritating
effect on the central nervous system.   He believed that the inflammatory reaction
of the lung protected the central nervous system from pathological changes
through interference with the diffusion of oxygen into the tissues.   There is
considerable variation between species and between individuals of the same

species in their susceptibility to oxygen. The minimum tension necessary to produce stimulation of the central nervous system is always well above that required to bring about pulmonary inflammation, but if the tension is sufficient the nervous symptoms develop rapidly and death may occur before there are any demonstrable changes in the lungs.

In 1903 Hill and McLeod (5) considered the question of the effect of compressed air on the respiratory exchange. From their experiments on mice they concluded, in agreement with an observation of Paul Bert, that a partial pressure of oxygen equal to 1 atmosphere does not increase, but rather lessens the processes of oxidation.

On the other hand, experiments in lower animal forms (6) have shown that the metabolism of many lower invertebrates, both marine and terrestrial, and some of the higher invertebrates such as the lobster and annelid worm is proportional over a wide range to the oxygen tension. This does not appear to be true of vertebrates.

Curiously enough, Lavoisier's original observation of the toxic effects of breathing high concentrations of oxygen at atmospheric pressure did not receive the attention of later workers (with the exception of Lorrain Smith) until Schmiedehausen (1) and David (7) confirmed his findings. They reported that pure oxygen supplied to a dog through a tracheal cannula produced a mild pulmonary hyperemia in as short a period as 15 minutes. 1 hour's exposure of a dog to 90 per cent oxygen in a chamber brought about definite hyperemia and extravasation of blood into the bronchi and alveoli. Similar results were noted in mice and guinea pigs, particularly after longer exposures. One guinea pig was exposed to 40 to 60 per cent oxygen at atmospheric pressure for 69 hours and 37 minutes. 6 hours after being taken out of the chamber into room air the animal died. Postmortem examination showed bronchopneumonia. This is the only case we have found recorded in the literature of pulmonary inflammation following exposure to increased oxygen tensions of less than 70 per cent of an atmosphere.

Benedict and Higgins (9), in 1911, carried out a series of experiments on the effect of inhalation of oxygen-rich mixtures for short periods of time on normal young men. With 40, 60 and 90 per cent oxygen they found no change in metabolism or respiration but a definite decrease in the pulse rate, which was more or less proportional to the percentage of oxygen breathed.

Bornstein and Stroink (10), in 1912, report the first case of experimental oxygen poisoning in man. Bornstein placed himself in a pressure chamber exposed to 2 atmospheres of pure oxygen. After 50 minutes he began to have cramps, first in the right, then in the left arm. The cramps ceased as soon as the pressure was lowered. These investigators also carried out a series of experiments on the effect of high oxygen tensions on dogs, apes, cats and rats. A dog and an ape, kept for several months in 0.6 atmosphere of oxygen, showed slight anemia,—an interesting observation in view of the opposite effect of low oxygen tensions.

Retzlaff (11), in 1913, found that the inhalation of oxygen produces vasoconstriction of the pulmonary blood vessels in the cat, and he suggests that the

beneficial effect of oxygen administration in cardiac failure with pulmonary edema may be ascribed to the improved pulmonary circulation thereby produced.

In 1916, Karsner (12) made an extensive study of the pathology of oxygen poisoning in rabbits, and recorded the time relationships of the various changes and gave a definition of the type of pneumonia occurring. He found that "80 to 96 per cent oxygen under normal barometric pressure produces in 24 hours, or more commonly 48 hours, congestion, edema, epithelial degeneration and desquamation, fibrin formation, and, finally, a pneumonia, probably of irritative origin," and described by him as a "fibrinous bronchopneumonia." He also found a certain degree of congestion in all of the abdominal organs which he believed to be secondary to the damage done to the pulmonary circulation. There were no demonstrable changes in the hematopoietic system other than congestion.

In a later work (13) Karsner and his coworkers showed that high partial pressures of oxygen are definitely inhibitory to the growth of certain strains of bacteria, while on other strains they may have no effect. The growth of pneumococcus was not inhibited by high oxygen tension.

Cleveland (14), in 1925, reported a series of ingenious experiments which indicated that oxygen in high concentrations is peculiarly toxic to intestinal protozoa. In cockroaches, for instance, the oxygen is 135 times as toxic for flagellates and 26 times as toxic for ciliates as for the host cockroaches. Similar effects are seen in termites and to a less marked degree in frogs, salamanders and goldfish, so that the intestinal tracts of these animals can be cleared of protozoa by simply exposing them to a sufficient oxygen tension. This test is impossible to carry out in warm blooded animals because the host is more susceptible than the parasite.

Barach (15), in 1926, published a series of carefully controlled experiments on the effect of oxygen-rich atmospheres on normal rabbits and on rabbits with pulmonary tuberculosis. He found that 60 per cent oxygen produced no effect on the general appearance, activity and weight or growth of normal rabbits over periods as long as 1 to 4 months. Furthermore, no gross or microscopic pathological change was observed for periods as long as 1 to 2 months. Attempts to increase the resistance of the pulmonary epithelium to atmospheres containing 80 to 85 per cent oxygen by previous exposure to lower concentrations were unsuccessful. In one case pulmonary edema followed the inhalation of 70 per cent oxygen for 12 days. On the basis of these observations Barach states that the highest concentration of oxygen compatible with safety for therapeutic use should be regarded as 60 per cent.

EXPERIMENTAL.

Our own studies on the toxic effects of oxygen began with observations on three dogs and three rabbits kept in an atmosphere of approximately 80 per cent oxygen. The animals were placed in cages in a large oxygen chamber used ordinarily for the treatment of pneumonia patients. In this chamber it was possible to maintain satisfac-

tory cleanliness and isolation of one animal from the other and to provide for the proper removal of carbon dioxide and moisture, and maintenance of fairly constant conditions of temperature, relative humidity and air motion. Daily observations of the weight, body temperature and behavior of the animals, and more frequent records of the room temperature, humidity and oxygen concentration were made. After a 4 day control period with the chamber open to the atmospheric air the doors were closed and oxygen was admitted into the chamber. The oxygen content of the chamber was raised at once to a little over 50 per cent of 1 atmosphere and then gradually, during the next 30 hours, to 80 per cent, where, with minor fluctuations, it was maintained throughout the remainder of the experimental period.

Experiments on Dogs.

The first abnormal sign noted in the dogs was the refusal of food. This was observed on the 3rd day after exposure to 70 to 80 per cent oxygen in Dog 1, a young, immature dog, on the 4th day in Dog 2, and on the 5th day in Dog 3. All of the dogs lost weight after the 3rd day. *Vomiting* occurred on the 5th day in Dog 1, on the 6th day in Dog 3 and was absent in Dog 2. *Drowsiness* was first recorded on the 6th day in all three dogs. *Labored breathing* was first noticed on the 6th day in Dog 3, on the 7th day in Dog 1 and was absent throughout in Dog 2. This respiratory distress became progressively worse during the rest of the experimental period. It had the character of slow, labored, deep breathing often associated with an apparent expiratory effort. There were no seemingly significant variations in the body temperature. Text-fig. 1 shows the variations in body weight and temperature in relation to O_2 and CO_2 content of the chamber.

After 8 days exposure to 70 to 80 per cent of an atmosphere of oxygen the dogs were removed from the chamber and samples of their arterial blood were drawn for oxygen analysis by puncturing the femoral arteries.

Dog 1 was markedly cyanosed. There was a cardiac arhythmia, shown by electrocardiograph to be the result of extrasystoles. Blood-stained froth was pouring from the mouth. Breathing was labored. At each inspiration the flanks were sucked in and expirations were grunting. When turned on its back respiratory distress became very great. Analysis of the arterial blood showed an oxygen saturation of only 40 per cent.

Dog 2 showed no evidence of anoxemia or respiratory distress. The arterial blood was 94.6 per cent saturated with oxygen.

Dog 3, like Dog 1, though far less markedly, manifested respiratory disturbance. Respirations were slow and labored. The flanks sucked in with inspiration and expiration was accompanied by a prolonged groan. There was, however, no definite arterial anoxemia, the blood being 93.7 per cent saturated with oxygen.

The three dogs were then immediately and painlessly killed by the intravenous injection of from 10 to 20 cc. of a saturated solution of magnesium sulfate.   This
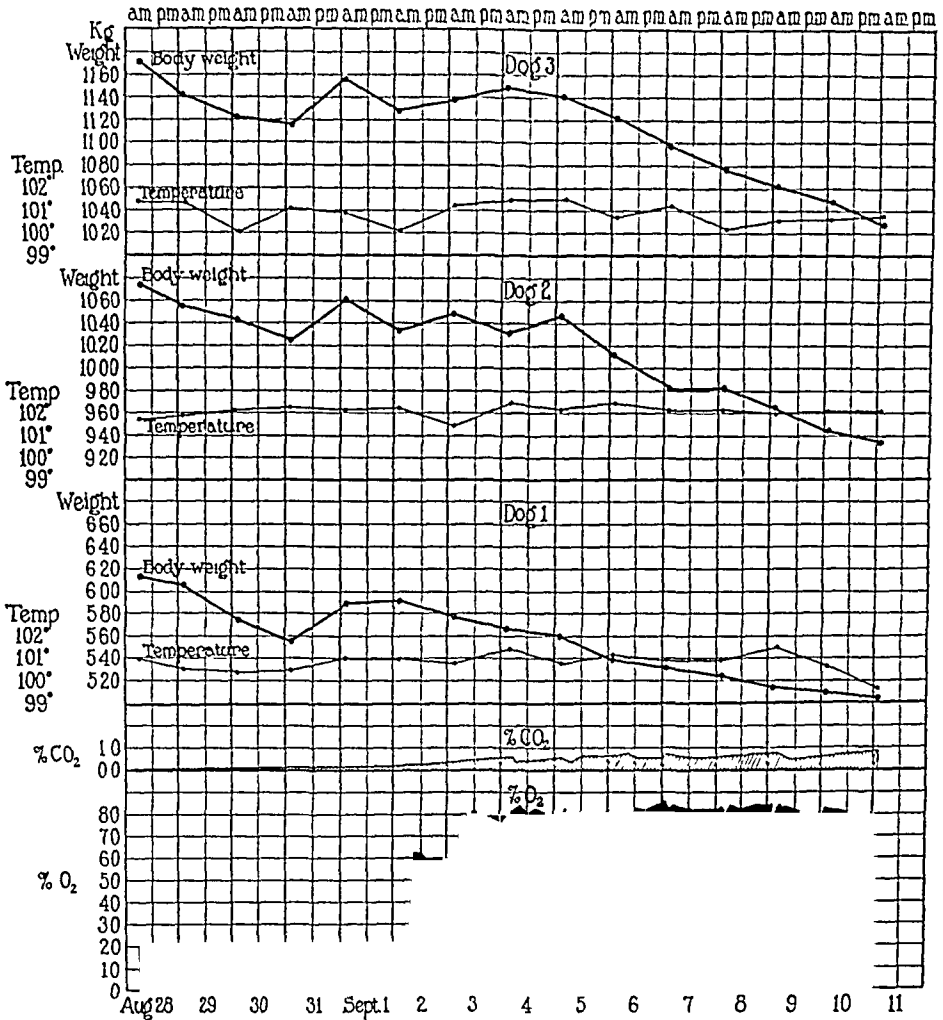
TEXT-FIG. 1. Chart showing loss of weight of three dogs confined in chamber containing 80 per cent oxygen.   The oxygen concentration is represented by the black area; the carbon dioxide by the narrow shaded area.   Carbon dioxide concentration remained below 0.8 per cent throughout the experimental period.

method is admirable for the study of pulmonary pathology, as the heart beat and respirations are arrested simultaneously and at once, unaccompanied by an ante-mortem struggle.

Autopsies on Dogs.

The gross and microscopic appearance of the lungs in the three dogs showed changes in keeping with the severity of their response to oxygen. When examined by a pathologist* who was unfamiliar with the symptomatic course of these three animals the lesions were graded in order of intensity thus: Dog 1, Dog 3, Dog 2, which will be seen to correspond to the manifestations cited above.

Description of Lesions in Lungs of Dog 1.

When the thorax was opened the lungs were found to be collapsed. They were mottled, beefy red—bright in some areas and dull red in others. There were no normally pink lobes. The trachea was full of blood-tinged froth. There was no free fluid in the pleural cavities. The lungs were disturbed as little as possible, except for punctures made for the purpose of making cultures. They were fixed *in situ* by distending them with Helly's fluid injected through a tube tied in the trachea. The roots were then ligated and the lungs carefully removed from the thorax and placed in Helly's fluid. Histological preparations were made by the usual paraffin technique, sections being cut at 7 μ and stained with Giemsa and hematoxylin and eosin. The sections revealed a general destructive process of a non-infectious character involving all parts of both lungs, the chief features being: (1) capillary engorgement with some hemorrhage; (2) the presence of interstitial and intraalveolar serum; (3) hypertrophy and desquamation of alveolar cells; and (4) interstitial and intraalveolar infiltration of mononuclear cells.

No microorganisms were seen in any of the sections examined. It will be shown later that these lesions, though not so marked in the dog, are pronounced in the lungs of the rabbits exposed to oxygen in the same chamber with the dogs. A photograph of a characteristic section of lung tissue from Dog 1 is shown in Figs. 1 to 3.

Description of Lesions in Lungs of Dog 2.

On opening the chest wall the lungs were collapsed and of essentially normal appearance, being uniformly coral-pink. There was no evidence of infiltration or consolidation; nor was there any free fluid in the pleural cavities. The lungs were treated in the same manner as those of Dog 1. Microscopic examination showed little change except for an apparent capillary engorgement.

Description of Lesions in Lungs of Dog 3.

Both lungs were collapsed. The right lung was, for the most part, normally pink in color, but there were a few small hemorrhagic areas near the periphery, especially on the anterior borders of the ventral lobe. The left lung had several small hemorrhagic areas on the surfaces of all lobes which were otherwise of normal

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* Dr. L. T. Webster of the Staff of The Rockefeller Institute, whom we wish to thank for his help in interpreting the nature of the lesions.

appearance. There was no free fluid in the pleural cavities. Microscopic examination showed capillary engorgement with some hemorrhage. Fig. 4 shows a photomicrograph of a characteristic region taken from the right lower lobe of Dog 3.

The gross appearance of all the other viscera was essentially normal.

Attempts at Cultivation of Bacteria.

Broth cultures taken from the heart's blood in all three dogs were sterile, and material from the lung punctures streaked on blood agar also showed no bacterial growth.

Experiments on Rabbits.

Three rabbits, housed in separate cages, were placed in the same chamber with the dogs just described, and exposed to identical atmospheric conditions as the dogs. Daily observations of body temperature and weight were made as well as careful inspection of behavior. No significant fluctuation in temperature occurred. All of the rabbits showed a slight loss of weight during the experimental period. *The first symptom of significance was marked dilatation of the alæ nasi accompanying respiration.* This was noticed on the 6th day after exposure to 70 to 80 per cent oxygen in Rabbit 1; on the 6th day in Rabbit 2; on the 5th day in Rabbit 3. From their general appearance at this time the prediction was made that they would die in the order in which they eventually did die, namely No. 3 first, then No. 1 and finally No. 2. The dilatation of alæ nasi was followed by definite signs of respiratory distress, gasping inspirations with the use of the accessory muscles of respirations, the mouth opening wide with each inspiration. This was accompanied by definite cyanosis of the tissues about the nose and of the ear tips. These symptoms appeared from the 6th to 7th day in all three animals. The intensity of the respiratory distress grew worse, the animals showing marked orthopnea when turned on their backs for the purpose of taking their temperatures. Rabbits 1 and 3 died on the 7th day after exposure to 70 to 80 per cent oxygen.

From the appearance of cyanosis and the low oxygen saturation found in the arterial blood of Dog 1 it was believed that the immediate cause of death was probably anoxemia resulting from the lesion of the respiratory membrane. To test this point further it was decided to take Rabbit 2 out of the chamber, with the anticipation of its being immediately asphyxiated by the sudden lowering of alveolar oxygen tension. When this was done the animal at once had a convulsive seizure and died in a few moments after exposure to atmospheric air.

Autopsies on Rabbits.

With minor variations in intensity of reaction the gross and microscopic appearance of the three rabbits' lungs were closely similar. All three showed mottled dull to beefy red discoloration—with much gross edema, and either froth or fluid in the trachea. Rabbit 1 had 2 to 3 cc. of blood-tinged fluid in both pleural cavi-

ties, which, on direct smear, showed many lymphocytes and leucocytes and fibrin, but no bacteria.   A few cc. of a similar but not blood-tinged fluid was found in the pleural cavities of Rabbit 2.   None was seen in Rabbit 3.

Microscopic examination of the lungs of the three rabbits showed in varying degrees of severity the same reaction as seen in Dog 1, though on the whole a more intense one, *viz.:* capillary engorgement with hemorrhage, interstitial and intra-alveolar serum, hypertrophy and desquamation of alveolar cells, interstitial and intraalveolar infiltration of mononuclear and of a few eosinophilic cells.   No microorganisms were found in any of the sections examined.   Photomicrographs of sections from the lungs of Rabbits 1 and 3 are shown in Figs. 5 and 6.   Heart's blood cultures were sterile.   With the exception of an occasional mold and colony of large spore-bearing bacillus most of the plates streaked with material obtained from lung puncture were likewise sterile.

Experiment on a Guinea Pig.

Protocol.—December 3.   A guinea pig was placed under a bell jar, so arranged on a wooden platform that oxygen could be blown into the jar at the desired rate. A basket containing soda-lime, for the removal of CO_2, was suspended in the jar. At 4.25 p.m. the flow of oxygen through the jar was begun at the rate of 1 liter a minute.

December 4.   The guinea pig looks normal.   There is no dyspnea.   Analysis of sample of gas in the bell jar: Oxygen, 99 per cent.   Carbon dioxide, 0.67 per cent.

December 6.   12 noon.   Respirations deep.

5 p.m.   No apparent dyspnea.   The guinea pig has eaten lettuce and some oats.

December 7.   9 a.m.   Found dead.   Autopsy showed the lungs to be a deep, dull red, resembling liver in appearance.   Sections of the lungs placed in 10 per cent formalin sank to the bottom of the container.

Experiments on Mice.

Four full grown mice were placed by pairs into two dialyzing jars which were found to be convenient receptacles for subjecting them to the desired gas mixtures. The jars were partly filled with wood shavings to serve as bedding for the mice and provide for their warmth.   Two of the mice acted as controls and two of them were to be subjected to a high concentration of oxygen.   Compressed air was run into the control jar from a pressure tank at a rate of 1 liter per minute and oxygen into the other.   This rate of flow provided for an adequate removal of CO_2 and moisture.   It was believed that any harmful influence which might result from the effect of compression and commercial handling of oxygen might be controlled by the use of air compressed to the same degree in cylinders identical to those containing the oxygen.

Protocol.

Oxygen Mice.

Mouse marked with picric acid. Weight 19.6 gm.

Mouse marked with carbolfuchsin. Weight 20 gm.

Nov. 16, '25, 4.30 p.m. O_2 flow started at 1 liter per min.

Nov. 17, '25. Mice appear normal. O_2 concentration in jar 98 per cent.

Nov. 19, '25. Mice appear normal. They are lively and restless. They remain on top of the shavings, whereas control mice are buried. O_2 concentration in jar 98 per cent. CO_2 0.39 per cent.

Nov. 20, '25. Mice appear normal. O_2 concentration 98.5 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25, 11 30 a.m. Mouse marked with picric acid is found dead. Weight 15 8 gm. Mouse marked with carbolfuchsin is gasping for breath. Its mouth opens wide and its whole body shakes at each inspiration. Cyanosis of tail, feet and nose. 2.45 p.m. Mouse dead. Weight 15.3 gm.

Control Mice.

Mouse marked with mercurochrome. Weight 25 gm.

Unmarked mouse. Weight 19 gm.

Nov. 16, '25, 4.30 p.m. Air flow started at 1 liter per min.

Nov. 17, '25. Mice active. Buried in shavings most of the time. No condensation of moisture on walls of jar.
Analysis of air in jar: O_2 = 22 09 per cent.
CO_2 = 0.22 per cent.

Nov. 18, '25. Mice appear normal.
Analysis of air in jar: O_2 = 21.58 per cent.
CO_2 = 0.25 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25. Mice appear normal.
Analysis of air in jar: O_2 = 21.68 per cent.
CO_2 = 0 4 per cent.
4.30 p.m. Compressed air tank nearly empty. Jar connected with house air pressure system and air run in at the same rate of flow, *viz.:* 1 liter per min.

Nov. 23, '25. Mice appear normal.

Nov. 24, '25. Mice appear normal.

Nov. 25, '25. Mice appear normal. Mouse marked with mercurochrome now weighs 27.6 gm. Unmarked mouse now weighs 20.6 gm. Jar cleaned and fresh wood shavings put in. Flow from compressed air cylinder again begun at rate of 1 liter per min.

Nov. 27, '25. Mice appear normal.

Nov. 28, '25, 8.40 p.m. Mice appear normal.

Nov. 29, '25, 10.30 a.m. Mice appear normal.

Nov. 30, '25. Mice removed from jar. They are active and normal in appearance. Mouse marked with mercurochrome weighs 27.1 gm. Unmarked mouse weighs 20.5 gm.

Autopsy of both these mice showed the lungs to be uniformly discolored a deep red, almost indistinguishable from liver. The lungs sank when placed in Zenker's fluid.

The experiments thus far reported demonstrate quite clearly, we believe, that for several mammalian species, the mouse, guinea pig, rabbit and dog, inhalation of commercial oxygen in concentrations greater than 70 per cent of an atmosphere may lead to a train of physiological changes consisting of drowsiness, loss of appetite, loss of weight, dyspnea and cyanosis, usually culminating in death from extreme oxygen want. The cause of the oxygen want is undoubtedly to be sought for in the acute pulmonary changes, to be characterized as a diffuse hemorrhagic edema of the lungs found in all these species.

Is it possible that this destructive process is due not to the oxygen itself but to some impurity in it introduced in the process of manufacture? The oxygen used thus far in this work was commercially prepared by a method now commonly employed, namely: the fractional distillation of liquid air. Before concluding that the pathological effect is due to oxygen and not to the presence of impurities, parallel experiments were done on mice kept in jars containing oxygen prepared in two differing ways: (1) by the so called "air reduction" process, (2) by the electrolytic dissociation of water.

This experiment shows that mice confined in a jar containing 96 to 98 per cent oxygen electrolytically prepared show the same physiological and pathological reactions as mice confined in equivalent concentrations of oxygen prepared by the "air reduction" process.

Burrows (16) reported the observation that oxygen was inhibitory to the growth of tissue cultures only when the gas had been led through rubber tubing, and believed that the toxic effect was due to a substance formed by the reaction of oxygen or ozone with rubber. Burrows, however, prepared his oxygen by the electrolysis of dilute H_2SO_4. By this method there is usually a contamination with ozone, which Burrows removed by bubbling the gas through olive oil. Under these circumstances it was not inhibitory to the growth of his culture material.

To control this factor two mice were placed in a dialysis jar connected with a tank of electrolytically prepared oxygen. The oxygen from the cylinder was led through glass tubes and bubbled successively through three wash bottles containing respectively 2M H_2SO_4, 2M NaOH and an approximately 10 per cent solution of K_2MnO_4. 4 days after the flow of oxygen was begun both mice were gasping for breath and incoordinated in their motions. The following day one of

Protocol.

Air Reduction Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked. O₂ flow started at 1 liter per min. 3.50 p.m.

Nov. 25, '25, 2.20 p.m. Both mice appear normal. O₂ concentration 96 per cent. CO₂ concentration 0.27 per cent.

Nov. 27, '25, 11.50 a.m. Mice appear drowsy and inactive. O₂ concentration 99 per cent. CO₂ concentration 0.41 per cent.

Nov. 28, '25, 8.40 p.m. Mice alive but very dyspneic.

Nov. 29, '25, 10.30 a.m. Safranine-marked mouse is dead. Second mouse living but very dyspneic. Moves about actively, however. Coat is ruffled. O₂ concentration 97 per cent. CO₂ concentration 0.11 per cent.

Nov. 30, '25. The surviving mouse breathing very rapidly, but not gasping. O₂ concentration 98 per cent.

Dec. 2, '25. Died at 11 a.m. Autopsy shows usual picture of red liver-like appearance of lungs. The removed lungs sink when placed in distilled water.

Electrolytic Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked. O₂ flow started at 1 liter per min. 3.50 p.m.

Nov. 25, '25, 12.30 p.m. Mice lively. Appear normal. O₂ concentration 96 per cent. CO₂ concentration 0.55 per cent.

Nov. 27, '25, 11.30 a.m. Mice appear normal, but drowsy. O₂ concentration 98 per cent. CO₂ concentration 0.50 per cent.

Nov. 28, '25, 8.40 p.m. Both mice very dyspneic.

Nov. 29, '25, 10.30 a.m. Severe dyspnea. 4.30 p.m. Unmarked mouse gasping. Lower jaw drops with each inspiration. Other mouse looks sick but is not gasping. Both move about when jar is shaken. Coats are ruffled. O₂ concentration 96 per cent. CO₂ concentration 0.18 per cent.

Nov. 30, '25. Both mice gasping. Noses and tails dusky bluish. Have eaten very little.

Dec. 1, '25. Both mice found dead. Lungs are deep purple-red the color of liver. Sink in water.

them was dead and the other, when moribund, was etherized. The lungs of both showed the usual changes.

In another experiment oxygen was bubbled through olive oil for the purpose of removing traces of ozone, even though its presence could not be shown by the starch-iodine test on the gas as it emerged from the cylinder. Mice exposed to this oxygen died of the same symptoms and showed the same autopsy findings as all the others described above.

DISCUSSION.

The phenomena described in this paper seem to have general application. The mammalian organism can survive exposure to oxygen concentrations varying roughly from 6 per cent to 60 per cent of an atmosphere. Beyond these limits it rapidly deteriorates, and at each extreme, death results from oxygen deprivation. At the lower limit oxygen want results from the diminished alveolar oxygen tension. At the upper limit, although alveolar oxygen tension is far above normal, the diffusion membrane of the lung is so damaged that in spite of the increased head of pressure in the alveoli, the arterial blood remains unsaturated, and the animal dies from anoxemia. This process possibly is of a "protective reaction" nature on the part of the organism, but going too far culminates in death. The organism, in its unsuccessful effort to achieve a new equilibrium, is kept alive by the very environmental condition which ultimately destroys it.

SUMMARY AND CONCLUSIONS.

1. Oxygen in concentrations of over 70 per cent of an atmosphere is poisonous to dogs, rabbits, guinea pigs and mice.
2. The poisonous effects manifest themselves in drowsiness, anorexia, loss of weight, increasing dyspnea, cyanosis and death from oxygen want.
3. The cause of oxygen want is a destructive lesion of the lungs.
4. The lesion may be characterized grossly as an hemorrhagic edema. Microscopically there is to be seen in varying degrees of intensity (a) capillary engorgement with hemorrhage, (b) the presence of interstitial and intraalveolar serum, (c) hypertrophy and desquama-

tion of alveolar cells, (*d*) interstitial and alveolar infiltration of mononuclear cells.

5. The type of tissue reaction is not characteristic of an infectious process and no organisms have been recovered at autopsy from the heart's blood or from lung puncture.

6. The poisonous effects of inhalations of oxygen-rich mixtures do not appear to be related to impurities in the oxygen, nor are they related to faulty ventilation, excessive moisture or increased carbon dioxide in the atmosphere of the chambers in which the experimental animals were confined.

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EXPLANATION OF PLATES.

PLATE 27.

FIGS. 1 and 2. Photomicrographs of sections of right lower lobe of lung of Dog 1. Magnification × 130. The pictures show the intraalveolar transudation of serum and red blood corpuscles.

FIG. 3. Section of right lower lobe of Dog 1. Magnification × 500. The picture shows desquamated alveolar epithelial cells.

PLATE 28.

FIG. 4. Section of lower lobe of right lung of Dog 3, showing engorged and tortuous alveolar capillaries. Magnification × 130. This probably represents the initial hyperemic stage before hemorrhage and edema have occurred.

FIG. 5. Section of lung of Rabbit 1. Magnification × 130. The picture shows transudation of serum and cells as well as cellular exudate.

FIG. 6. Section of lung of Rabbit 3. Magnification × 500. The picture shows desquamation of alveolar epithelial cells as well as an exudate consisting chiefly of mononuclear and a few eosinophilic cells.
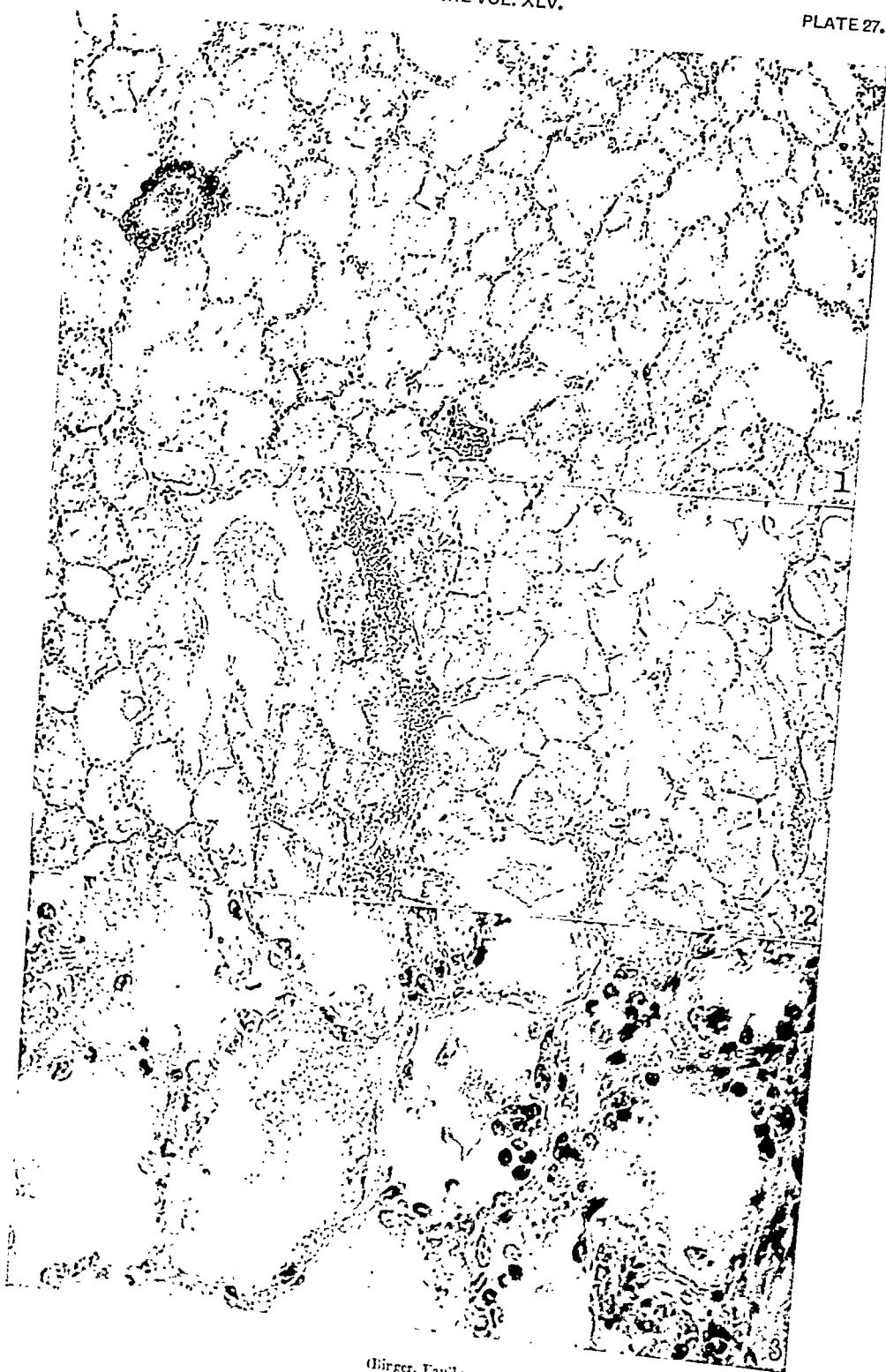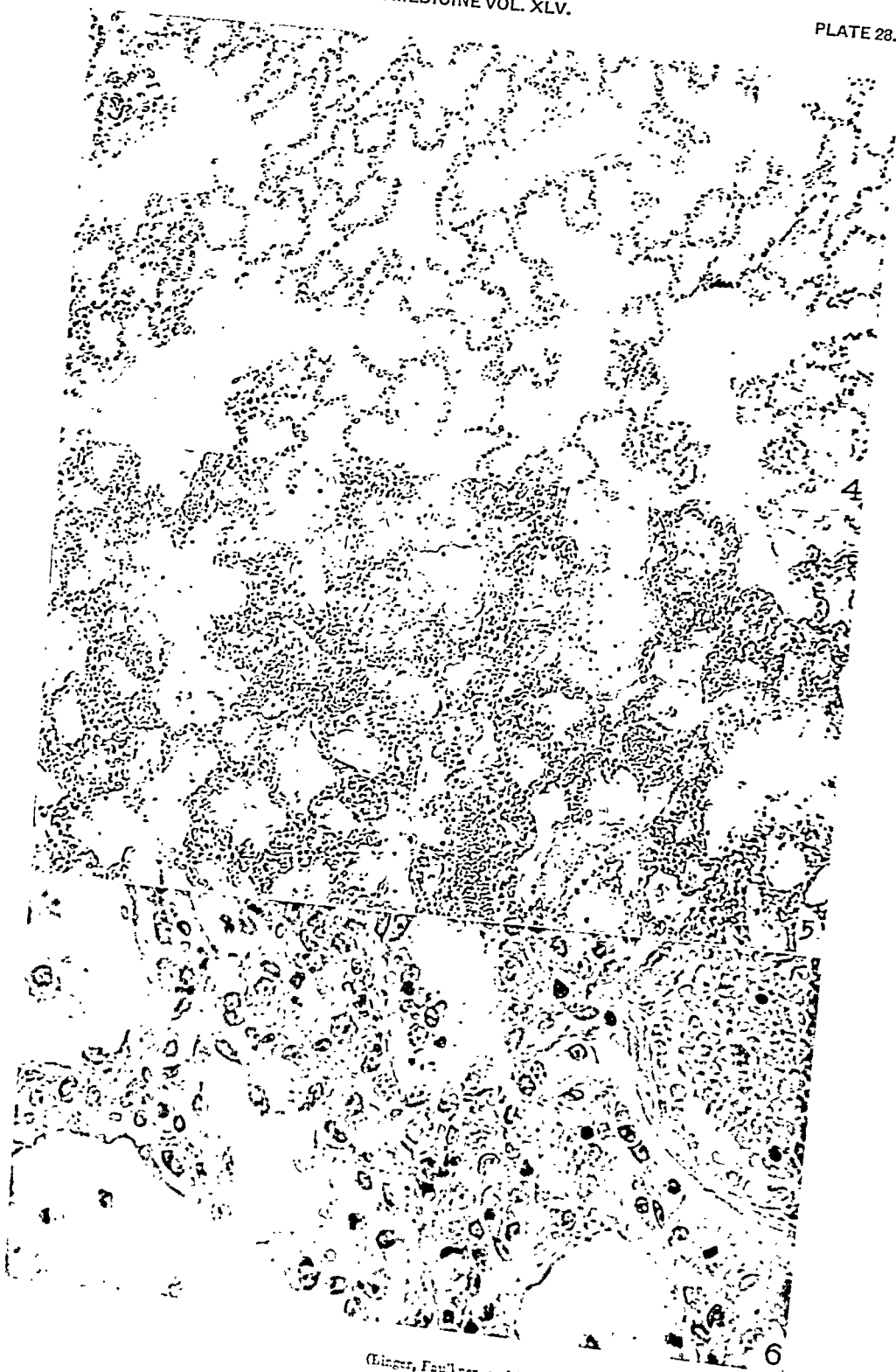
OXYGEN POISONING IN COLD BLOODED ANIMALS.

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PLATE 29.

INTRODUCTION.

In another paper (1) the effect of breathing atmospheres rich in oxygen on a variety of mammals was reported, and the nature of the injury to the lungs and the cause of death was discussed. It was pointed out that the pulmonary lesion was characterized by capillary dilatation and hemorrhagic edema. The work here to be described was undertaken not simply for the purpose of extending the study to other species but with the hope of observing the effect of high concentrations of oxygen on a capillary system more accessible to inspection than that in the lungs. For this purpose we utilized the web of frogs exposed to high concentrations of oxygen (90 to 99 per cent) over long periods. The web capillaries were examined periodically during life and after death sections were made of the skin, as well as of the lungs, heart, liver and kidney. No changes were observed in the capillaries or in the pigment cells as a result of oxygen administration. Two frogs grew definitely darker in color after 7 and 10 weeks exposure, respectively, but we attach no special significance to this, because color changes in the frog's skin are readily produced by a variety of causes difficult to control (2). In this instance inanition may have been a cause, since the frogs were not fed during the course of the experiment.

Krogh (3) has shown that the cutaneous respiratory exchange of the frog differs from its pulmonary respiration in that carbon dioxide is eliminated chiefly through the skin, while oxygen is absorbed chiefly by the lungs. Enough oxygen may be absorbed through the skin, however, to keep a frog alive for several weeks at 7-8°C. when the pulmonary ventilation is entirely cut off (4). This may be

accomplished by applying a gag to the frog in such a manner that it is unable to close its mouth, thus inhibiting the normal swallowing movements, whereby this animal forces air into its lungs (5). By placing gagged frogs in jars containing 90 to 95 per cent oxygen it was hoped to bring about changes in the skin capillaries, since the oxygen absorption would, under these circumstances, be chiefly through the skin and pharyngeal mucous membrane. None of the frogs showed any changes, however, in the skin capillaries.

These experiments, though negative with respect to the original subject of enquiry, brought out the fact that frogs could survive, apparently unharmed, in concentrations of oxygen poisonous to mammals. This observation seemed of sufficient interest to merit further study. Did it signify that cold blooded animals are resistant to the poisonous action of high concentrations of oxygen? And is the difference in their behavior from the behavior of mammals solely dependent on their differences in body temperature?

EXPERIMENTAL.

Experiments on Frogs.—Two frogs placed in a shallow dish containing a little water were continuously exposed under a bell jar to an atmosphere with an oxygen concentration averaging from 80 to 90 per cent. After 21 days exposure they were removed from the jar. Throughout the experiment and at the time of removal from the oxygen they remained apparently normal in appearance and behavior. The frogs were killed and autopsied. No gross changes in the lungs or other viscera were to be seen. Two other frogs were kept continuously in 90 to 98 per cent oxygen for respective periods of 49 and 68 days without apparent damage to their well being. At the close of the experiment they were found to be more deeply pigmented than they had been at the start, forming a marked contrast in color to two control frogs. No other changes in them were to be seen. Mammals exposed to similar environmental conditions would probably have died within the 1st week. To discover whether this difference in behavior was due to differences in body temperature an effort was made to keep frogs alive at incubator temperature (37.5°C.), but the frogs were unable to withstand this and died within 24 hours. For this reason we decided to make experiments on turtles whose life habits frequently expose them to high temperatures.

Experiments on Turtles.—Is the turtle also resistant to high concentrations of oxygen?

Protocol. Experiment 1.—A common adult pond turtle (*Chelopus guttatus*) was placed in a shallow dish containing a little water. The dish was covered with a

bell jar through which a stream of oxygen was allowed to run at the rate of 1 liter per minute. After 23 days exposure to an atmosphere containing an oxygen concentration for the most part above 90 per cent the turtle was removed in an apparently normal state. At autopsy the lungs were found to be normal in both gross and microscopic appearance (see Fig. 1).

Experiment 2.—The above experiment was repeated on another adult of the same species. After 10 days in an atmosphere of 90 per cent oxygen the turtle was removed from the bell jar in an apparently normal state.

Experiment 3.—Five young green turtles (*Chrysemes elegans*) lived for 3 weeks in 97 to 99 per cent oxygen at room temperature (23–26°C.) without appearing in any way affected by the unusual environment. A sixth member of this group was found dead after 17 days exposure, but there was no respiratory distress observed, and no definite evidence of pulmonary pathology was seen. These experiments indicate that the turtle, like the frog, can survive, unharmed, in concentrations of oxygen fatal to mammals.

Effect of Mammalian Temperature on Turtles.

Two adult specimens of *Chelopus guttatus* survived for 36 days in an incubator at 37.5°C., showing at no time loss of appetite or other signs of ill health.

An adult *Chrysemes elegans* spent 34 days, and six young individuals of the same species spent 21 days in the incubator. At the end of this time they were all lively and eating well.

From these observations it can be concluded that turtles are unharmed by prolonged exposure to room air warmed to 37.5°C.

Effect of Combination of High Concentrations of Oxygen and Mammalian Temperature on Turtles.

To find out whether the difference in the reaction of turtles exposed to high concentrations of oxygen from that of mammals was dependent upon their differences in body temperature, we exposed turtles to 90 per cent oxygen warmed to 37.5°C. Individuals of various ages and species were confined in an ordinary bacteriological incubator in which the oxygen concentration was kept above 90 per cent by blowing a stream of oxygen through it. It was soon learned that under these conditions turtles behaved like mammals in their susceptibility to oxygen, exhibiting like abnormalities in function and structure. Since young turtles behaved somewhat differently from adults the experimental results will be classed accordingly.

Experiments on Adult Turtles.

Protocol. Experiment 4.—May 22, 1926. An adult male pond turtle (*Chelopus guttatus*), kept in a shallow dish containing a little water, was put into an ordinary bacteriological incubator. This individual had previously survived, without ill effect, a 36 day exposure to room air warmed to 37.5°C. The incubator was provided with a pan of soda-lime for absorption of carbon dioxide, and a constant stream of oxygen was now run through the incubator at the rate of 1 liter per minute. Analysis at 11 p.m. showed the oxygen percentage to be 94 and the temperature 37.5°C.

May 23, 11 a.m. Oxygen concentration in the incubator 91 per cent.

May 24, 9.45 a.m. Oxygen concentration in the incubator 94 per cent. CO_2 concentration in the incubator 0.02 per cent.

At about 11 a.m. it was noticed that the turtle was opening its mouth to breathe. It stretched its neck, opened its mouth and extended its fore legs at each inspiration. These gasping breaths increased in frequency during the afternoon.

May 25. At 10 a.m. the turtle was found dead. Autopsy showed the lungs to be opaque and blood-red instead of being of the normal pale gray transparency. Microscopic examination showed marked engorgement of the pulmonary capillaries.

Experiment 5.—May 5, 1926. Another adult turtle of the same species (*Chelopus guttatus*) was placed in an incubator containing 90 per cent oxygen, as in the experiment just reported.

May 6, 9.10 a.m. O_2 concentration 93 per cent.

7.45 p.m. O_2 concentration 94 per cent. Temperature in incubator 37.5°C. Turtle rather inactive and refuses to eat an earthworm which is offered to it.

May 7, 12.30 p.m. O_2 concentration 95 per cent. Temperature 37°C.

May 8, 11 a.m. O_2 concentration 97 per cent. Temperature 38°C.

May 9, 12.30 p.m. O_2 concentration 95 per cent. Temperature 38.5°C.

May 10, 10 a.m. O_2 concentration 95 per cent. Temperature 38.5°C. The turtle shows a peculiar form of breathing today. It stretches its neck, then opens its mouth for several seconds and swells out its throat. It then shuts its mouth, retracting and deflating its neck, apparently forcing air into the lungs. This process is repeated about every 30 seconds.

May 11, 10.30 a.m. O_2 concentration 95 per cent. Temperature 38°C. Turtle still shows same type of breathing at a somewhat more rapid rate than yesterday. Accompanying each inspiratory gasp it extends its fore legs, as if to aid respiration.

May 12, 9.40 a.m. O_2 concentration 94.5 per cent. Temperature 37°C. Same type of breathing persists.

May 13, 9.30 a.m. Oxygen cylinder emptied during night. O_2 concentration fell to 42 per cent. Gasping for breath.

10 a.m. O_2 concentration 97 per cent.

1.15 p.m. Turtle apparently lifeless—lying with its head under water. Reflexes were still present, however. The plastron was removed and the heart was

found to be still beating. All the organs except the lungs were removed. These were left *in situ* and fixed in 10 per cent formalin. The lungs were definitely redder than normal. Microscopic examination showed a very marked vascular congestion with great dilatation of capillaries. An illustration of this may be seen in Fig. 2, which should be compared with Fig. 1, a section made from the normal turtle lung.

Experiments similar to the two just described were repeated on another individual of the same species, on two adult Cumberland terrapins (*Chrysemes elegans*) and on one box turtle (*Cistudo carolina*) approximately 15 years old. All of them ended fatally. The duration of life and the degree of pulmonary congestion found at autopsy varied in the different individuals.

These experiments show that high concentrations of oxygen, when combined with incubator temperature (37-38°C.), are fatal to adult turtles of several different species, producing changes analogous to those found in the mammal, namely: progressive dyspnea, culminating in death, with hemorrhagic changes in the lungs. Neither high concentrations of oxygen, nor warm room air alone brought about these changes.

Experiments on Young Turtles (Chrysemes elegans).

It has already been stated that young individuals of the species *Chrysemes elegans* survived unharmed exposure to 97 to 99 per cent oxygen for a prolonged period. They were found to be equally resistant to exposure to warm air, behaving in these respects just like the adults.

On exposing them to oxygen warmed to 37.5°C., it was observed that they survived longer than the adult specimens did. Eventually, however, they succumbed, showing similar hemorrhagic extravasations into the lungs.

Experiment 6.—Five young turtles (*Chrysemes elegans*), whose shells measured about 3.5 cm. in diameter, were exposed in an incubator at 37.5°C. to an oxygen concentration varying from 94 to 98 per cent of an atmosphere. After 21 days exposure, the first one died. After 24 days, a second died. After 27 days, two of the survivors showed curious wing-shaped dark areas on their backs, found at autopsy to be due to seepage of extravasated blood from the lungs. These two died respectively on the 32nd and 41st day after exposure. The lungs at autopsy were found to be very red, with extravasation of blood into the extrapulmonary tissue.

The last turtle died on the 50th day after exposure to warm oxygen. At autopsy its lungs were found to be red and edematous, with blood stains through the plastron and carapace.

DISCUSSION.

An adequate explanation for the facts presented in this paper is not at hand. It is clear that increasing the temperature does not increase the oxygen concentration in the body. The implication would seem to be that a reaction occurs between oxygen and pulmonary tissue whose temperature coefficient is such that it progresses at the temperature of the mammalian body but not at ordinary room temperature. Whether a chemical substance is produced which acts as an irritant to the lungs cannot be stated, or whether the increased temperature acts by raising the metabolic rate, which is known to enhance the toxic effect of certain substances.

SUMMARY AND CONCLUSIONS.

1. Exposure of frogs to atmospheres containing approximately 95 per cent of oxygen is without apparent effect on their state of well being, and produces no noticeable changes in the appearance of their web capillaries.

2. Turtles exposed to similar atmospheres are also apparently unaffected unless the oxygen be warmed to mammalian temperature.

3. At this temperature (37.5°C.) the turtles behave like mammals, showing loss of appetite, shortness of breath, death and, at autopsy, hemorrhagic extravasations in the lungs.

4. Young turtles are more resistant (or adaptable) to this change in environment than mature ones.

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EXPLANATION OF PLATE 29.

FIG. 1. Section of normal turtle lung showing thin walled respiratory membrane. Magnification × 240.

FIG. 2. Section of lung taken from turtle killed by exposure to warm oxygen. Magnification × 240. The photograph shows a characteristic hemorrhagic area with extravasated nucleated red blood corpuscles.

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PLATE 29.
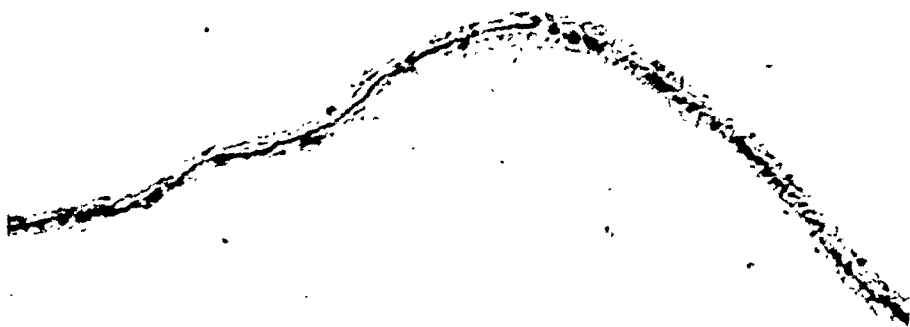
STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VII. On the Particulate Nature of Bacteriophage.

By J. BRONFENBRENNER, Ph.D.

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So called bacteriophage, as ordinarily obtained by filtration of broth cultures of susceptible bacteria after the completion of lysis, represents a heterogeneous mixture containing in addition to the active principle, the unaltered ingredients of the nutritive medium, products of bacterial metabolism, complex materials synthesized by bacteria, and liberated into the medium as the result of lysis of bacteria, etc.

Since at least a part of these substances is present in true solution, and since the active principle in all probability is carried by the particles of colloidal size (1), dialysis and differential ultrafiltration may offer means of eliminating from the filtrate the most highly dispersed and soluble ingredients. This is particularly advisable since we have found that such ingredients of the medium as inorganic salts, for example, may greatly increase the deleterious effect of various reagents which might be used in further purification of the active principle (2, 3). Numerous attempts to dialyze the active filtrates have given conflicting results (4–10). Not only have the findings differed when investigators employed membranes prepared of different material, but even when membranes of similar nature were used. Thus, for instance, Stassano and de Beaufort (11) found that phage passes freely through the membranes prepared from solutions of collodion in acetic acid, though such membranes were impervious to enzymes. Bechhold (6), as well as Villa (12), on the other hand, found that phage was kept back completely, even by such comparatively permeable membranes as those prepared from a 2 per cent solution of collodion in acetic acid. Our own findings show that even the more impervious membranes, up to 7 per cent and over, still allow diffusion of an appreciable fraction of the lytic agent.

However, when lytic filtrates were dialyzed through collodion membranes against fresh distilled water under osmotic pressure, it was found that while in general the rate of diffusion of phage was greater with the more permeable membranes, in no instance was it possible to observe a complete diffusion of the active principle. In all cases only a fraction of the active principle placed inside the membrane could be recovered in the dialysate, irrespective of the length of time during which dialysis was continued. So that if the dialysate was collected daily and replaced by fresh distilled water, after a few days the active principle no longer appeared in the outer fluid, while the inner fluid continued to show the presence of the bulk of the original active agent.

The following experiment is selected to illustrate this phenomenon.

Dialysis of Phage through Collodion under Osmotic Pressure.

Membranes of cylindrical shape were prepared from collodion dissolved in the mixture of alcohol and ether (13). By careful control of the initial amount of collodion used, the size of the surface over which it was spread, the time during which the solvents were allowed to evaporate from the film of collodion prior to its immersion in water, and the rate at which this evaporation of solvents was allowed to proceed, it was possible to prepare membranes of desired permeability. Membranes which were prepared under similar conditions were fairly comparable to one another in their permeability to water. The latter was determined in terms of time necessary for a given amount of water under constant pressure to pass through a unit of surface of the membrane suspended in air (13). By this procedure it was possible to check the relative permeability of membranes prepared and to select for duplicate experiments comparable membranes. Each membrane thus selected was filled with and suspended in water, and sterilized in the autoclave.

One of these membranes, allowing passage of 0.1 cc. of water under a pressure of 10 cm. of mercury over $\geqslant$ 44 sq. cm. of surface in 30 seconds, received 30 cc. of the Berkefeld filtrate of a lysed broth culture of *B. coli* (*coli* phage), was mounted on and suspended by means of a rubber stopper (through which was inserted a long glass tube 5 mm. in diameter) in a sterile receptacle containing 2000 cc. of sterile distilled water. After 24 hours of dialysis, the level of the water in the

Protocol I.

Dialysis of Phage under Osmotic Pressure.

		Duration of dialysis in days											Controls	
		0	1	2	3	4	5	6	8	10	12	15	Original fluid (inside)	Residue after dialysis (inside)
Osmotic pressure (about) in cm...		0	64.5	89	113.5	115.0	118.5	112.5	101.5	94	82	65		
Results of titration of lytic activity in cc.	10^{-10}	−	−	−	−								−	−
	10^{-9}	−	−	−	−								+	+
	10^{-8}	−	−	−	−			−	−	−	−		+	+
	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	+	+
	10^{-6}	−	−	−	−	−	−	+	−	−	−	−		
	10^{-5}	−	−	+	+	+	+		−	−	−	−		
	10^{-4}	−	−	+	+	+	+							
	10^{-3}	−	−	+	+									
	10^{-2}	−	+	+										
	10^{-1}	−	+	+										
Number of units of phage dialyzed per day			2×10^{8}	2×10^{10}	2×10^{9}	2×10^{8}	2×10^{8}	2×10^{4}						
Total number of units of phage													$\gtrsim 2 \times 10^{10}$	$\gtrsim 3 \times 10^{10}$ * $\gtrsim 1 \times 10^{10}$ †

$\gtrsim 3 \times 10^{10}$ (Original fluid control)

+ = lysis. − = no lysis. *By titration. †By calculation.

glass tube rose to the height of 64½ cm. At this time the fluid in the outer re-
ceptacle was removed and replaced by fresh, sterile, distilled water. This first
fraction of dialysate was titrated for its lytic activity by Appelmans' method, and
the results of the titration were recorded, as shown in Protocol I. After the ex-
piration of a second period of 24 hours, the osmotic pressure was recorded, the
dialysate was again removed and titrated (Protocol I, 2), and the outer container
was again filled with fresh, sterile, distilled water.

A similar procedure was followed at intervals indicated in Protocol I for 15
days, at the end of which time the experiment was terminated and a sample of the
solution inside the collodion bag was titrated, in addition to the sample of the
outer fluid.

It is apparent that over 50 per cent of the active principle was lost
into the outer fluid during the first 5 days of dialysis, but none came
through after this.[1]

These findings suggest at first glance that the particles which carry
the active principle might not be uniform in size, and that the larger
particles were kept back, while the smaller ones came through. How-
ever, since lytic filtrate contains a great deal of colloidal material
(notably proteins), it is also possible that the changes in the rate of
diffusion of phage were caused by a gradual diminution in the perme-
ability of the membrane, due to adsorption of these substances (23).
While regularity of the rate of change in osmotic pressure throughout
the experiment would in itself suggest that there was no abrupt change
in the permeability of the membrane about the 5th day of dialysis,
still this possibility had to be considered before drawing conclusions
as to lack of uniformity in the size of particles carrying active prin-
ciple, particularly as earlier publications lead one to believe that the
active principle consists of particles of approximately uniform size,
approaching 20 $\mu\mu$ in diameter (11, 6, 14, 8).

Possibility of the Partial Occlusion of the Pores of the Membrane.

In order to exclude this possibility, the following experiment was
performed.

30 cc. of a fresh lytic filtrate was dialyzed through one of two collodion mem-
branes, the permeability of which was similar to that of the membrane used in the

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[1] The titration of the activity of the residue does not indicate this loss because
of the tenfold method of dilution in titration.

preceding experiment. The outer fluid was changed daily and was found to be free from active principle from the 7th day on. On the 12th day[2] 20 cc. of the residue within the dialyzing bag was removed and placed into a second bag. At the same time, 20 cc. of fresh lytic filtrate was introduced into the first bag. Both were suspended in distilled water and dialysis was continued for 4 days, as illustrated in Protocol II.

These results indicate that the residue remaining inside the dialyzing membrane does not diffuse through a fresh membrane of similar

Protocol II.

Permeability of the Membrane after the 6th Day of Dialysis.

	Dialyzing Bag I 30 cc. of fresh lytic filtrate	
Titer of the dialysate in cc.	1st day 1×10^{-4}	
	2nd " 1×10^{-6}	
	3rd " 1×10^{-7}	
	5th " 1×10^{-4}	
	6th " 1×10^{-2}	
	7th " Negative	
	8th " "	
	9th " "	
	10th " "	

On the 12th day 20 cc. of the fluid inside the bag was removed and replaced by 10 cc. of fresh lytic filtrate. The fluid removed was placed into Bag II.

	Dialyzing Bag I		Dialyzing Bag II
	Residue from dialysis + 10 cc. of fresh lytic filtrate		20 cc. of the residue from Bag I
Titer of the dialysate in cc.	1st day 1×10^{-4}		Negative
	2nd " 1×10^{-4}		"
	3rd " 1×10^{-5}		"
	4th " 1×10^{-3}		"

permeability, and that, on the other hand, the first membrane which held back a portion of the active principle after 5 days of dialysis is permeable to the fresh lytic filtrate. The inference is that the failure of the active principle to appear in the dialysate after 5 days (Protocol I) is not due to the change in permeability of the membrane, but

[2] 2 days were allowed to elapse in order to obtain the final check on the titration of the dialysate removed on the 10th day.

to the fact that particles carrying the active agent are not uniform in size, and that only smaller particles could pass through the pores of the membrane used in this experiment.

If the above inference is correct—that is, if the fraction of the active principle which dialyzed through is carried by particles of smaller size—then the active principle present in dialysate must pass freely through another membrane of similar permeability. In view of the fact that the dialysate, as obtained in the preceding experiment, is very highly diluted and does not lend itself therefore to such an experiment as the one just outlined, we undertook to fractionate the lytic filtrate by ultrafiltration instead of dialysis as before.
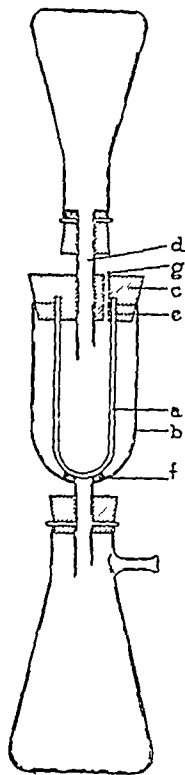
FIG. 1.

The preparation of the membranes and the procedure followed in ultrafiltration are as follows:

Alundum thimbles (Norton RA 360 135 mm. × 45 mm.), glazed to our specifications for a distance of 2 cm. from the rim on the inside surface and for a distance of 2.5 cm. on the outer surface, were used to support a collodion membrane. The latter consists of soluble cotton of any desired concentration, in glacial acetic acid, and is deposited on the inner surface of the alundum under a vacuum. For this purpose a clean (preferably freshly incinerated) thimble (a) is filled with water and adjusted to a Gooch funnel of proper size, (b) by means of a rubber stopper (c) having a small hole in the center (d) and a circular groove fitting the ridge of the thimble (e). The funnel is then placed over a filtration flask connected with the vacuum pump. On the bottom of the funnel rests a thin, perforated, rubber disk (f), protecting the lower end of the thimble against possible injury due to pressure against the funnel (Fig. 1). After all free water is removed by suction, and only traces of water remain adsorbed on the wall of the thimble, a solution of collodion is admitted to the thimble through the hole in the center of the stopper until the thimble is filled with collodion. The suction is maintained for 60 seconds so as to remove all the air from the pores of the thimble and to secure an even membrane without flaws. As soon as connection with the vacuum pump is discontinued, the collodion is quickly poured out of the thimble while the latter is being rotated, to secure an equal thickness of the membrane. During this manipulation care must be taken in handling the thimble not to touch the unglazed surface, to prevent the

production of flaws in the membrane due to uneven evaporation of acetic acid. When no more collodion runs off (2~3 minutes) the thimble is placed under a water faucet and quickly filled with water in order to solidify the collodion. Special precautions must be taken not to. wet the outer surface of the thimble before filling it with water, as deposition of water droplets on the outer surface drives the air contained in the clay inward and results in the production of air pockets between the clay wall and the membrane. When the entire thimble is filled with water it may be dropped into a vessel containing running water, for the purpose of washing out the acetic acid. The latter process can be hastened by placing the thimble into the funnel and washing it under negative pressure with warm water.

Protocol III.

Permeability of Collodion to Whole Phage and to Ultrafiltered Fraction Respectively under Negative Pressure.

Titer in cc.	Original phage (control)	Ultra-filtrate A	First washing B	Second washing C	Third washing D	Residue E	Fourth washing F	Ultra-filtrate from A G	Ultra-filtrate from B H
10^{-10}	−					−			
10^{-9}	+					+			
10^{-8}	+					+			
10^{-7}	+		−			+			−
10^{-6}	+	−	+			+			+
10^{-5}	+	+	+			+		−	+
10^{-4}	+	+	+	−		+		+	+
10^{-3}	+	+	+	+		+		+	+
10^{-2}	+	+	+	+	−	+	−	+	+
10^{-1}	+	+	+	+	+	+	+	+	+
10^{-0}	+	+	+	+	+	+	+	+	+

+ = lysis.   − = no lysis.

If the removal of acid is not complete, a portion of the active agent is adsorbed on the membrane during ultrafiltration. Similar loss of the active agent is observed if, instead of adequate removal of acid, the latter is merely neutralized with ammonia (6). It is preferable, therefore, to remove the last traces of acid by adequate washing and electrodialysis (21). Membranes prepared in this way may be used repeatedly, provided they are kept under water when not in use and are sterilized by immersion in alcohol.[3]

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[3] In another connection Dr. Hetler and myself have used this ultrafilter for continuous filtration of large amounts of material by inserting a tube connected with an inverted container through the hole (d) in the center of the stopper. In this case a second hole (g) which serves to admit the air operates as an automatic regulator for the continuous filling of the thimble from the reservoir above.

Passage of Phage through Collodion under Pressure.

20 cc. of fresh bacteriophage solution was forced, under vacuum of 38 cm., through a membrane of 7 per cent soluble cotton in acetic acid. The ultrafiltrate thus obtained was collected and titrated to determine the concentration of the lytic agent in it (Protocol III, A).[4] The residue which failed to pass through the membrane was diluted with 20 cc. of distilled water and subjected to a second filtration through the same membrane. The ultrafiltrate was again collected and titrated (Protocol III, B), and the residue again diluted with 20 cc. of water and forced through the membrane for the third time. As will be seen from the protocol (Protocol III, C), the ultrafiltrates obtained at this time, as well as upon a further repetition of the procedure (Protocol III, D), contained only faint traces of active agent. The residue in the thimble was again diluted to 20 cc. with water, and a sample taken for titration showed that the residue still contained the bulk of the lytic agent (Protocol III, E). The contents of the thimble were then subjected to filtration through the same membrane for the fifth time, and the ultrafiltrate titrated (Protocol III, F). At this time the ultrafiltrate collected in the first filtration (Protocol III, A) was added to the residue in the thimble, and subjected to ultrafiltration. It will be seen (Protocol III, G) that the fluid which came through the membrane at this time contained a comparatively high concentration of active principle.

At the same time the ultrafiltrate obtained from the first washing of the residue (Protocol III, B) was forced through a fresh 7 per cent membrane and the ultra-filtrate thus obtained was titrated. In this case (Protocol III, H), the ultrafiltrate showed no diminution in activity, as compared with Solution B.

The results of this experiment confirm those obtained earlier, in that they show that only a portion of lytic agent passes through the membrane, that the residue which is held back contains the bulk of the active principle, and yet it can be washed repeatedly without loss of any more active substance into the filtrate. On the other hand, the filterable portion of the active principle passes freely when added to this residue, or when filtered through a fresh membrane.

These findings suggest that the active principle is carried by the particles, which may differ in size, and therefore that only a part of the active principle which is adsorbed on the smallest particles may pass through a membrane of given porosity.

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[4] Titrations were made both by the method of serial dilution and by the plaque method. Incidentally it was observed that ultrafiltrates consistently yielded plaques considerably smaller in size than those produced by the agent remaining on the filter.

These experiments were repeated a number of times with the lytic agents active against *B. coli*, *B. dysenteriæ* Shiga, *B. pestis caviæ*, and staphylococcus, and in all cases identical results were obtained. It was found that with all lytic filtrates the proportion of filterable fraction was approximately the same, irrespective of the origin of bacteriophage or bacterial substratum. This observation suggested that nutrient broth itself served as a principal source of the particles capable of passing through the ultrafilter. The validity of this inference seems to be supported by the following experiment, in which the addition of sterile broth to the non-filterable residue of the phage resulted in renewed passage of the active principle through the membrane.

Effect of Broth on the Filterability of Phage.

A portion of lytic filtrate was subjected to ultrafiltration and six repeated washings with water as previously (Protocol III). The ultrafiltrates thus obtained were collected and subsequently titrated for lytic activity (Protocol IV, A).

At the same time another portion of the same phage was subjected to ultrafiltration and three repeated washings with water. At this time the residue in the thimble was diluted to 20 cc. with sterile broth (pH = 7.4) and subjected to ultrafiltration. Following this it was washed with water as before two more times (Protocol IV, B). As a control a third portion of the phage was filtered through a third membrane in exactly the same manner as the second but instead of broth in the fourth washing a buffer mixture of pH = 7.4 was used (Protocol IV, C).

It appears that when sterile broth was added to the residue on the filter (Protocol IV, B), a portion of the phage which was adsorbed on the coarser particles became detached and came through the collodion with the filterable portion of the broth, thus apparently assuming a more dispersed state. This conclusion, however, is valid only if it can be shown that the broth did not modify the membrane itself. This was actually done. When a clean ultrafilter was saturated with broth prior to the filtration of the residue, the latter did pass through to some extent. However, if such an ultrafilter was washed free of broth, it became again impermeable to the phage residue, which would not have been the case if broth had been adsorbed on the membrane in a manner analogous to that of gelatin in the experiments of Hitchcock (23).

Protocol IV.

Effect of Broth on the Rate of Ultrafiltration of Phage.

Membrane	Sample	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}
	Original phage	−	+	+	+	+	+	+	+	+	+	+
7 per cent membrane A	Ultrafiltrate			−	−	−	+	+	+	+	+	+
7 per cent membrane A	First washing with water			−	−	+	+	+	+	+	+	+
7 per cent membrane A	Second washing with water					−	−	+	+	+	+	+
7 per cent membrane A	Third washing with water							−	−	+	+	+
7 per cent membrane A	Fourth washing with water								−	−	+	+
7 per cent membrane A	Fifth washing with water									−	−	+
7 per cent membrane A	Sixth washing with water									−	−	+
7 per cent membrane B	Ultrafiltrate			−	−	+	+	+	+	+	+	+
7 per cent membrane B	First washing with water			−	−	+	+	+	+	+	+	+
7 per cent membrane B	Second washing with water				−	−	−	+	+	+	+	+
7 per cent membrane B	Third washing with water					−	−	−	−	+	+	+
7 per cent membrane B	Fourth washing with broth	——→			−	−	+	+	+	+	+	+
7 per cent membrane B	Fifth washing with water				−	−	+	+	+	+	+	
7 per cent membrane B	Sixth washing with water					−	−	−	+	+	+	+
7 per cent membrane C	Ultrafiltrate				−	−	+	+	+	+	+	+
7 per cent membrane C	First washing with water				−	−	+	+	+	+	+	+
7 per cent membrane C	Second washing with water				−	−	+	+	+	+	+	+
7 per cent membrane C	Third washing with water						−	−	+	+	+	+
7 per cent membrane C	Fourth washing with buffer (pH = 7.4)							−	−	+	+	+
7 per cent membrane C	Fifth washing with water								−	−	+	+
7 per cent membrane C	Sixth washing with water									−	−	+

Titer of activity in cc.

DISCUSSION.

Direct (20), as well as indirect (6) microscopic examination of lysed cultures of bacteria, and especially the behavior of lytic filtrates in high dilutions (1) and their ability to cause the appearance of discrete foci of lysis in bacterial cultures on solid media have led to the generally accepted view that the active principle of transmissable lysis (bacteriophage) is present in filtrates of lysed cultures in the form of discrete particles.

By means of ultrafiltration through membranes of known porosity several authors (6, 11, 14) have independently estimated the size of these particles as being approximately 20 $\mu\mu$ in diameter, and consequently having a calculated mass of about $\frac{4}{3}\pi$ 10^{-18} gm. (15). Moreover, similar figures were obtained when the size of particles of bacteriophage was estimated by optical methods. Thus, von Angerer (16) found, on the basis of his study of the turbidity of filtrates, that the diameter of particles approximates 20–35 $\mu\mu$. This uniformity of the size of the particles, as found by different investigators and by means of different methods, has in itself appeared to many to be a strong indication in favor of the conception that the particles represent the units of an autonomous organized virus, as originally suggested by d'Hérelle.

However, some experiments, particularly those showing that the active principle is capable of spreading from the focus radially (16), independently of the multiplication of bacteria, and independently of gravity, and that the rate of its spread is conditioned by the density of the medium (1) seem to militate against ready acceptance of such a view.

If the autonomous particulate nature of the active principle is accepted, such spreading must be accounted for either by postulating for the particles the power of locomotion (17), or by assuming that they secrete a diffusible enzyme (18). But independent locomotion, even if claimed to exist (17), (whether it be ameboid or due to the activity of a propelling mechanism, such as flagellum or cilia) would explain only the transport of a given particle in one plane at the time, whereas the active principle spreads from the focus in all directions

simultaneously (16). The secretion of the active enzyme has actually been suggested by d'Hérelle (18), but the evidence presented by him was later found to be inadequate (19).

On the other hand, such radial spreading could be easily accounted for by a working assumption (3) that the observed particulate distribution of phage is only apparent and is due to its ready adsorption on coarser colloidal particles of the medium. That such an assumption is more valid has been suggested by our experiments in which the number of particles endowed with specific activity of phage in a given volume of a filtrate was altered, depending on changes in the degree of dispersion of colloids in the medium (1). Experimental data presented in this paper strengthen this conception further by showing that the particles present in filtrates of lysed cultures of bacteria and endowed with properties of the phage are not uniform in size. Only that portion of the active agent which is carried by the smallest particles was found capable of passing through semipermeable membranes, while the bulk of it failed to pass through even on repeated washing under pressure. While this failure to pass through a membrane is not of itself sufficient evidence to indicate the size of the particles, under the conditions of our experiments, where the effect on the membrane was excluded by proper controls (Protocol III, G and H), failure of the active residue to pass the filter appears to indicate that it is composed of particles too great in size to pass through the largest pores of the ultrafilter employed.

In this connection, it is of interest to note that in our earlier experiments (19), it was found that only a portion of the active agent was carried down with the precipitate produced by the addition of an excess of alcohol at 7°C., and the supernatant fluid usually retained enough active agent to make 1×10^{-5} cc. of it capable of causing lysis of susceptible bacteria. If the rate of precipitation was increased by the addition of electrolytes or by an increase in temperature, the precipitate carried down more of the active principle. It would appear that in this case also only larger particles were precipitated by the alcohol at 7°C., and the supernatant fluid represented that portion of the phage which corresponds almost quantitatively to that fraction which passed the ultrafilter in the experiments reported in this paper.

The fact that active principle can thus be shown to be distributed in the medium in the form of particles of different size does not necessarily deny its autonomous particulate nature, since the phage can conceivably be assumed to be a pleomorphic virus. However, the fact that the addition of broth to the non-filterable residue (presumably composed of units of the agent of larger size) allows it to pass through the membrane which held it back before the addition of broth, is contrary to such an assumption, provided, as we have shown, the permeability of the membrane was not altered by the addition of broth.

Incidentally, in the light of these findings it seems likely that used Berkefeld candles which might have retained some active principle might not give it up into the filtrate if water is passed through them, and yet, if broth is passed instead of water, the filtrates may show some activity due to the detachment of the phage from the coarser particles by the broth. Such an effect of broth would explain the occasional findings of several workers (22) who believe they have caused spontaneous production of phage by filtration of bacterial cultures, heated lytic filtrates, and even sterile broth—substrata assumed by them to be free from bacteriophage before filtration.

SUMMARY AND CONCLUSIONS.

When filtrates of lysed cultures (bacteriophage) are subjected to prolonged dialysis under osmotic pressure against water, the presence of the lytic agent can be detected outside the membrane only during the first few days. The residue remaining inside the membrane contains the bulk of the original lytic agent, and yet it is no longer capable of diffusing into the outer solution.

The interruption of diffusion is shown not to be due to any alteration in the permeability of the membrane. Moreover, the residue fails to diffuse through a fresh membrane of similar permeability, while the dialyzed portion of the phage passes quantitatively through a new membrane. When ultrafiltration under pressure was substituted for dialysis, the residue on the filter could be washed repeatedly with water without giving off into the filtrate any more active agent. However, if broth was substituted for water, a renewed diffusion of the active agent resulted.

These results are interpreted as indicating that the colloidal particles present in the lytic filtrates (and apparently endowed with properties of bacteriophage) do not represent autonomous units of the active agent, but merely serve as a vehicle on which the agent is adsorbed. They vary in size within limits wide enough to permit fractionation by means of ultrafiltration.   When the coarser particles retained by the ultrafilter are washed with broth, some of the active agent is detached from its coarse vehicle particles.   This agent, now more highly dispersed, is capable of passing the filter which held it back previously.

Preparation of a simple ultrafilter used in these experiments is given in detail.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VIII. The Mechanism of Lysis of Dead Bacteria in the Presence of Bacteriophage.

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With but few exceptions (1, 2), those engaged in the study of bacteriophage agree that one of the essential features of transmissible lysis of bacteria is the fact that the lytic principle undergoes an increase in activity exclusively in the presence of living and actively multiplying bacteria. Without active growth of susceptible bacteria, there is no reproduction of lytic agent and no observed bacterial lysis. Although dead susceptible bacteria readily adsorb the lytic agent, they do not dissolve (3–7). However, Gratia and Rhodes (8) observed that dead staphylococcus may be slowly lysed by the bacteriophage, and that the concentration of the latter in solution probably increases during this process. If live staphylococci are present simultaneously, the lysis of the dead bacteria is more rapid. The lytic effect of live on dead staphylococcus was observed by them also in the absence of bacteriophage[1] and was assumed by them to be due to the utilization of dead bacteria by the live in the process of nutrition (10). The relation of this phenomenon to the one described earlier by these authors (8) was not made clear. A year later Twort (11) independently observed the lysis of dead staphylococcus in the presence of bacteriophage and live bacteria, and suggested that this was made possible through the activation of the bacteriophage by some auxiliary substance contributed by the live bacteria. Because of the importance of these observations on the question of the mechanism of transmissible lysis, we undertook to study it.

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[1] A similar observation has also been made recently by Duran-Reynals (9).

The Lysis of Dead Bacteria.

An 18 hour agar slant culture of *Staphylococcus aureus* "G" was suspended in saline, sealed in an ampoule, and submerged for 1 hour in a water bath at 60° C. to kill the bacteria. 0.3 cc. of this suspension of dead bacteria was placed into each of a series of tubes containing 9 cc. of broth each, and an homologous bacteriophage alone,[2] or together with live staphylococci, was added to some of these tubes, as indicated in Protocol I.

The degree of turbidity resulting, as well as a subsequent increase or decrease in turbidity, was measured by comparison with a specially prepared set of standards in which the turbidity gradually increased from the first to the tenth tube. The figures expressing the results of this and later experiments indicate the serial numbers of the tubes in this arbitrary set of standards to which the turbidity in a given tube was comparable.

Protocol I.

Lysis of Dead Staphylococci.

Tube No	1	2	3	4	5	6
Broth in cc.	9	9	9	9	9	9
Suspension of dead staphylococci in cc.	0.3	0.3	0.3	0.3	0	0
" " live " " "	0	0	0.1	0.1	0.1	0.1
Phage in cc.	0	0.1	0	0.1	0	0.1
Salt solution	0.7	0.6	0.6	0.5	0.9	0.8

Incubated at 37°C.

Turbidity of resulting suspensions	Immediately	4	4	5	5	1	1
	After 4 hrs	4	4	8	4	8	0
	" 24 "	4	4	10+	1	10+	0

It will be seen from Protocol I that the suspension of dead staphylococci did not undergo lysis by the bacteriophage alone (Tube 2), but when both live staphylococci and the phage were present simultaneously (Tube 4), dead staphylococci underwent almost complete lysis, as shown by the decrease in turbidity of the suspension from that corresponding to Tube 5 of the standard scale to that corresponding to Tube 1—the lowest tube in the scale.

In attempting to repeat this experiment, using other bacteria than staphylococcus (*B. coli* and *B. dysenteriæ*), we failed to obtain clear-

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[2] Unless otherwise stated, the bacteriophage used in these experiments was taken out of the stock kept on ice several weeks.

cut results.   This explains the negative results obtained by Doerr and Grüninger (7), who attempted to produce dissolution of dead colon bacillus in the presence of corresponding bacteriophage and of live bacteria.   Moreover, we found that even with staphylococcus, reproducible results can be obtained only if the relative bulk of dead bacteria used is taken into account, as will be shown in the following experiment.

Effect of Variations in Relative Concentration of Phage and of Dead Bacteria.

Two series of eight tubes each received equal amounts of broth (5 cc.).   Each of the tubes of the first series (A) received 0.1 cc. of antistaphylococcus phage diluted in broth 1:1000; and the tubes of the second series (B) each received 0.1 cc. of the same phage diluted 1:100,000.   Following this, the first four tubes of each series received 0.1 cc. each of bacterial suspension containing 80 million of live staphylococci per cc., and gradually decreasing amounts of a suspension of dead staphylococci containing 220 million bacteria per cc.—the first tube of each series receiving 5 cc. of this suspension, the second 0.5 cc., the third 0.05 cc. respectively.   The fourth tube served as control and received no dead bacteria. The fifth, sixth, and seventh tubes of each of the series received respectively 5 cc., 0.5 cc., and 0.05 cc., of the suspension of dead bacteria only and no live bacteria, and the last tube (No. 8) of each series received no bacteria at all.   The volume of liquid in all the tubes was brought to 10.2 cc. with physiological salt solution, and the whole was kept for 24 hours at 37°C.   Changes in turbidity due to lysis of bacteria were recorded, as indicated in Protocol II, and after 24 hours' incubation all the tubes were placed for 30 minutes into a water bath kept at 56°C., and the phage titer of each mixture was determined by the method of Appelmans.

This experiment indicates that both in the series where the initial titer of the phage was 1×10^9 cc. (Protocol II, B, Tube 8), and where it was 1×10^{-1} cc. (Protocol II, A, Tube 8), dead bacteria alone adsorbed the entire phage (Tubes 5, 6, and 7 of each series), the rate of adsorption apparently depending on the number of dead bacteria present.   When the concentration of dead bacteria was comparatively low, the adsorption of phage was so slow that when live bacteria which had been added began to multiply, there was sufficient phage left free in solution to produce its usual effect on the live bacteria and to regenerate.   In the case where the initial concentration of phage was lower (Series B), the regeneration of phage took place only in Tube 3, containing the lowest number of dead bacteria.   In Series A,

Protocol II.

Effect of the Number of Dead Bacteria Present in the Mixture on the Regeneration of Phage.

	A								B							
Tube No	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Broth in cc.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Phage 1:1000 in cc.	0.1	0.1	0.1	0.1					0.1	0.1	0.1	0.1				
" 1:100,000 in cc.					0.1	0.1	0.1	0.1					0.1	0.1	0.1	0.1
Suspension of live staphylococci in cc.	0.1	0.1	0.1	0.1	5	5	5	5	0.1	0.1	0.1	0.1	5	5	5	5
" " dead " "	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0
Salt solution in cc.	0	4.5	5	5	0.1	4.6	5.1	5.1	0	4.5	5	5	0.1	4.6	5.1	5.1
Incubated at 37°C. for 24 hrs. The changes in turbidity during the incubation were recorded at intervals, as indicated below.																
Turbidity — Immediately	10	7	5	1	10	7	5	0	10	7	5	1	10	7	5	0
Turbidity — After 4 hrs.	10+	7	4	1−	10	7	5	0	10+	10	5	1−	10	7	5	0
Turbidity — " 24 "	10+	5	2	0	10	7	5	0	10+	10+	3	0	10	7	5	0
All tubes were heated at 56°C. for 30 min. and the titer of phage in each determined.																
Phage titer in cc.*	0	10^{-6}	10^{-7}	10^{-7}	0	0	0	10^{-7}	0	0	0	10^{-7}	0	0	0	10^{0}

* The sign 0 = no phage present in 1 cc. of the mixture.

Variation between 10^{-6} cc. and 10^{-7} cc. in the final titer is due to particulate distribution of phage (25).

where the initial concentration of phage was ten times greater, the regeneration of the phage took place both in Tube 3 and in Tube 2. It is evident that the absence of regeneration of phage in Tube 1 of Series A, and in Tubes 1 and 2 of Series B was due to the fact that the phage present in the mixtures was so completely taken up by the dead bacteria that by the time live bacteria had begun to multiply actively (which is essential for the regeneration of phage) after a period of initial lag, there was no free phage left in the mixture. As a result of this, the bacteria in these tubes remained intact, as is evidenced by the fact that the original turbidity of the contents increased slightly, due to multiplication of the live bacteria. On the contrary, in such tubes of each series as exhibited regeneration of phage in the presence of dead bacteria (Tubes 2 and 3 of Series A, and Tube 3 of Series B), the initial turbidity decreased, due to dissolution of dead bacteria.

Specificity of Lysis of Dead Bacteria.

It was seen in the preceding experiments that while phage alone does not cause lysis of dead bacteria, the latter are lysed if phage and live bacteria are present simultaneously and if the concentration of dead bacteria is kept sufficiently low not to interfere with the process of regeneration of the phage. Another condition essential for the lysis of dead bacteria is that the dead and live bacteria be of the same species. This requirement has already been indicated by Twort (11), and we have been able to confirm it. Dead colon bacilli or dead dysentery bacilli were not lysed in the presence of live staphylococcus and staphylococcus phage.

Time Relation of the Lysis of Live and of Dead Bacteria.

The preceding experiments show that live bacteria contribute some specific active principle necessary for the production of lysis of dead bacteria. Twort (11) has suggested that bacteria supply some sort of a complementary substance which activates the phage and disappears as the phage ages. In order to see whether such a substance is present, and to determine more accurately at what stage of lysis of live bacteria it first appears and how long its activity continues, the following experiment was performed.

A series of six tubes containing 10 cc. of broth each received 0.1 cc. of a suspension of live staphylococcus and 0.1 cc. of phage, and were placed in the incubator at 37°. The first tube of the series received at the same time 0.2 cc. of a suspension of heat-killed staphylococcus. At intervals of 2, 4, 6, etc., hours after

Protocol III.

Dissolution of Dead Bacteria Added during and after the Completion of the Lysis of Live Bacteria in the Presence of Bacteriophage.

	Test proper						Controls				
Tube No..................................	1	2	3	4	5	6	7	8	9	10	11
Broth in cc..................	10	10	10	10	10	10	10	10	10	10	10
Suspension of live staphylococci in cc......................	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0
Phage (filtrate) in cc..........	0.1	0.1	0.1	0.1	0.1	0.1	0	0.1	0	0.1	0

Incubated at 37°C.

Interval before addition of the suspension of dead bacteria in hrs.....................	0	2	4	6	24	48					
Suspension of dead bacteria in cc......................	0.2	0.2	0.2	0 2	0 2	0.2	0	0	0.2	0.2	0.2
Turbidity	Immediately......	4						1	1	4	3	3
	2 hrs. later......	4	4					1	1+	4	3	3
	4 " "	5	3	4				5	1	6	3	3
	6 " "	3	2	2	3			10	0	10	3	3
	24 " "	1	2	6	1	3		10+	0	10+	3	3
	30 " "	1+	3	6	1+	2−		10+	1	10+	3	3
	48 " "	6	6	7	7	3*	7*	10+	4	10+	3	3
	72 " "					6*	6*	10+	6	10+	3	3

* Final results in these tubes are somewhat obscured, due to increase in turbidity caused by the multiplication of the resistant bacteria, but the lysis of dead bacteria has taken place even in these tubes, as suggested by the temporary diminution in turbidity in Tubes 5 and 6.

the beginning of the experiment, other tubes of the series each received in turn 0.2 cc. of the same suspension of dead bacteria. The progress of lysis of live, as well as of dead bacteria, was followed and is recorded in Protocol III in terms of turbidity, by comparison with a standard scale, in which the higher numbers indicate greater turbidity. A fall in turbidity below that of the control tube (No. 11), containing dead bacteria alone, indicates the lysis of dead bacteria. It will be

seen that the initial fall in turbidity in all the tubes of the series (Nos. 1 to 6) is followed by a subsequent increase. This is due to the growth of resistants following the initial lysis of the susceptible live bacteria, so that the results in Tubes 5 and 6 are difficult to interpret on the basis of turbidity.

The results show that dead bacteria are dissolved when added to the live bacteria undergoing lysis, any time within 24 hours after the beginning of the experiment, and probably even later, irrespective of the stage at which the lysis of live bacteria may happen to be. Since the lysis of live bacteria under the conditions of the experiment was completed in the first 6 hours (see Tube 8), and since dead bacteria were dissolved when added even much later, it is evident that the actual lysis of live, is not essential for the dissolution of dead bacteria, and that the products of such lysis alone are capable of causing dissolution of dead bacteria.

However, we have observed that filtrates of lysed cultures which should thus contain these products are inactive against dead bacteria (Protocol I). It was suspected, therefore, that during filtration they might have been kept back by the filter, while phage was able to pass freely. If such were the case, then it would appear that the agent responsible for the dissolution of dead bacteria is distinct from the phage proper and can be separated from the latter because of the difference in their respective diffusibility.

In order to determine if such were the case, an attempt was made to interpose a semipermeable membrane between the live and dead bacteria during the lysis of the former to see whether this procedure would prevent the dissolution of the dead bacteria (12).

Separation of Lysed Cultures into Two Fractions by Means of a Semipermeable Membrane.

A series of cylindrical collodion membranes 15 mm. in diameter and 50 mm. long were prepared under carefully controlled conditions. After hardening in water, these membranes were tested for their relative permeability by measuring the time required for 0.1 cc. of water, under pressure of 10 cm. of mercury, to be forced through the membrane suspended in air. It was found that membranes allowing this amount of water to pass through in from 20 to 30 seconds were suitable for the experiment. A number of such selected collodion bags were filled with and suspended in water, and sterilized in the autoclave for 10 minutes, at 20 pounds pressure. While the autoclaving renders the membrane somewhat less permeable

Protocol IV.

Separation of Phage Proper from the Agent Responsible for the Lysis of Dead Bacteria by Dialysis.

	A Inside	A Outside	B Inside	B Outside	C Inside	C Outside	D Inside	D Outside
Broth in cc....................	5	150	5	150	5	150	5	150
Phage "	0 5		0.1	0.1		0.1	0.1	0.1
Live bacteria..............								
Dead "					0 1	0.1	0.1	
Duration of incubation in hrs......	0	5	0	5	0	5	0	5
		24		24		24		24
Results — Gross lysis { Inside....	0		0		0	0	0	0
{ Outside..				Lysis	0	Lysis		Partial lysis
Phage titer in cc. { Inside....	1×10^{-8}	1×10^{-7}			0	1×10^{-8}		
{ Outside..	1×10^{-5}	1×10^{-7}			1×10^{-8}	1×10^{-5}		
	0							
Bacteria in millions per cc. { Inside....					163	165	267.5	96*
{ Outside..					161		29	

*These figures represent averages of several counts made on Breed plate or in a counting chamber.

to water, we have found by experience that bags selected and tested as stated above are uniformly permeable to bacteriophage. At the time of the experiment the dialyzing thimbles were removed from the water in which they had been sterilized, filled with measured amounts of sterile broth, and placed into suitable containers, with aseptic precautions. In each experiment four dialyzing thimbles were set up as follows:

I. In the first dialyzing unit a measured amount of antistaphylococcus bacteriophage was added to the broth inside of the dialyzing thimble, and a small portion of the resulting dilution of phage was immediately taken out for titration by the method of Appelmans. At the same time a small portion of the broth outside of the bag was likewise removed for titration, and the whole unit was placed in the incubator at 37°C. After an interval of 5 and 24 hours respectively, the titration of the fluid inside and outside the thimble was carried out, and it was found that the lytic agent dialyzed freely under these conditions ((Protocol IV, A).

II. In the second dialyzing unit 5 cc. of broth were placed inside and 150 cc. of broth outside the thimble as before, following which 0.1 cc. of antistaphylococcus bacteriophage was added to the broth inside the thimble, and 0.1 cc. of an 18 hour old culture of susceptible staphylococcus to the broth outside. The whole unit was placed in the incubator and observed at intervals. It was found that live bacteria placed outside the thimble underwent lysis (Protocol IV, B), just as they do when placed in direct contact with phage without the interposition of the membrane.

III. The third dialyzing unit was set up exactly as the preceding one, except that both live bacteria and the phage were placed outside, and on the inside only 0.1 cc. of a suspension of dead (heat-killed) staphylococcus. As the experiment proceeded in the incubator at 37°C., lysis of live bacteria took place outside the thimble, and the phage dialyzed into the thimble, where it was demonstrated by titration. However, the actual count of dead bacteria placed into the thimble remained unaffected throughout the experiment (Protocol IV, C).

IV. In the fourth dialyzing unit the phage was placed outside the dialyzing thimble and allowed to dialyze for 6 hours into the sterile broth inside. At this time 0.1 cc. of a suspension of live staphylococcus was added to the fluid inside the thimble, and a sample was removed for immediate count, which was found to be 1,000,000 bacteria per cc. Immediately following the removal of the sample, 0.1 cc. of a suspension of dead staphylococcus was added, and again a sample was removed. The bacterial count in this sample indicated the presence of 267,500,000 bacteria per cc. Thus, the initial mixture inside the thimble was composed of 1,000,000 live, and 266,500,000 dead bacteria per cc. From then on the bacterial count was repeated at intervals, and it was found that at the end of 5 hours it had fallen to 29,000,000 per cc., and at the end of 24 hours it had risen to 96,000,000 per cc., due to overgrowth of resistant bacteria. As the initial mixture contained 266,500,000 dead bacteria per cc., it is evident that at the end of 5 hours, practically 90 per cent of the dead bacteria had been dissolved.

These tests indicate that the phage responsible for transmissible lysis of live bacteria is easily diffusible, but that the agent liberated during the lysis of live bacteria and causing dissolution of dead bacteria does not pass through the membrane, so that dead bacteria remain unaffected if the active lysis of live bacteria takes place on the other side of the membrane, even though the bacteriophage can be demonstrated in abundance in the dialysate in which dead bacteria are suspended.

Protocol V.

Effect of Adsorption with Live Bacteria on the Phage Titer and Power to Dissolve Dead Bacteria of Lysed Staphylococcus Cultures.

	A						B
	Dissolution of dead staphylococcus						Titer of phage
Original lysed culture in cc.......	0	5					1×10^{-6}
Supernatant fluid after the first adsorption in cc.....			0	5			1×10^{-3}
Supernatant fluid after the second adsorption in cc....... . .					0	5	1×10^{-1}
Broth in cc.....	5	0	5	0	5	0	
Suspension of dead bacteria in cc .	0 2	0 2	0 2	0 2	0 2	0 2	

Covered with toluene and incubated at 37°C.

Turbidity {	Immediately .. .	3	3	3	3	3	3
	After 6 hrs..	3	3−	3	3−	3	3−
	" 24 "	3	1	3	2−	3	1

Separation, by Adsorption, of the Phage from the Agent Dissolving Dead Bacteria.

If freshly lysed cultures of staphylococci contain two active agents, as suggested by the experiment just described, it should be possible to separate them from each other also, by removing the phage from the solution through adsorption on live susceptible bacteria, and leaving in the solution only the agent dissolving dead bacteria.

Accordingly, cultures of Staphylococcus "G" were subjected to lysis by the appropriate bacteriophage, at 37°C. At the same time mass cultures of staphylococcus were grown on the surface of agar in Blake bottles. The next day bacteria

collected from the surface of three Blake bottles were washed by centrifugation, and to the solid mass of bacteria at the bottom of the centrifuge tube were added 20 cc. of lysed staphylococcus culture. At this time bacteria were suspended in the fluid above, by vigorous shaking, and the whole mixture was placed on ice for 2 hours, to allow for adsorption of the phage on bacteria. At the completion of a 2 hour period, the bacteria were thrown down by centrifugation at a high speed, for 1 hour. The centrifuge used for this purpose was supplied with a cooling device, so that lysis of bacteria was prevented during the experiment. A portion of the supernatant fluid was removed for examination of its bacteriophage content, as well as of its ability to dissolve dead bacteria, and to the remainder of the fluid was added another lot of bacteria collected from three Blake bottles. The mixture was shaken, placed on ice for 1 hour, and again bacteria were separated by centrifugation. The supernatant fluid was again tested as before. The results of these titrations are recorded in Protocol V.

As the results of this experiment indicate, it is possible to remove most of the bacteriophage proper by adsorption on bacteria, without affecting the power of the lysate to dissolve dead bacteria.

Dissolution of Dead Bacteria by Filtrates of Lysed Cultures in the Absence of Live Bacteria.

The last two experiments show that lysis of dead bacteria depends upon the presence in the freshly lysed cultures, in addition to the phage proper, of another active agent which does not go through the semipermeable membrane of collodion. This agent may conceivably be held back by the porcelain during filtration, and thus the fact that lytic filtrates, as usually prepared, do not cause the dissolution of dead bacteria becomes explainable. However, we found that the retention of this agent by the filter was complete and constant, while Gratia and Rhodes (8), report that the filtrates exhibit a certain amount of activity against old as well as against dead staphylococcus cultures.

Since the failure of lytic filtrates (bacteriophage) to dissolve old or dead bacteria constitutes, in our opinion, a characteristic which assumes fundamental importance in an attempt to understand the mechanism of transmissible lysis, we felt that it was necessary to determine beyond any doubt whether in the experiments of Gratia the active filtrates contained only the phage, or whether, under the conditions of his experiments, a certain amount of the second agent also had passed into the filtrate. Apart from the possibility that the

efficiency of the filters used by Gratia and ourselves might have been different, it seemed likely, that if large amounts of lysed cultures are filtered through a given filter, its efficiency may gradually decrease, and the substance, which at first is held back, may appear in the filtrate after continued filtration, thus possibly explaining the activity of his filtrates (26).

In order to test this possibility, a flask containing 1 liter of broth received a suitable amount of the suspension of the 18 hour agar growth of staphylococcus and of bacteriophage. The resulting mixture was distributed equally into three smaller flasks and incubated at 37°C. for 2, 4, and 6 hours respectively. At the end of 2 hours' incubation, one of the flasks was taken out and immediately subjected to fractional filtration through a new Berkefeld V candle, under pressure of 60 mm. of mercury as follows: At first 50 cc. of liquid were removed from the flask and filtered. The filtrate was collected into a sterile receptacle. Then a second 50 cc. portion of the contents of the flask was filtered through the same candle and the filtrate collected into a second receptacle. Then followed a third fraction of 50 cc. and so on—five fractions in all being employed. At this point the filter candle was discarded and the five fractions of the filtrate were immediately subjected to examination for sterility,[3] phage content, and for their power to cause lysis of dead staphylococcus, as indicated in Protocol VI, Section 1 A. At the proper intervals the contents of the other two flasks were similarly filtered, each through its own new filter candle, and the fractional filtrates thus obtained were examined, as indicated in Sections I B and I C of Protocol VI.

As another possibility which could explain discrepancies in the results it seemed to us of interest to inquire also into the rate of deterioration suffered by the agent responsible for the lysis of dead bacteria, under the influence of heat and preservation. In order to elicit the rôle of these factors, all the fractional filtrates (immediately after the removal of a small fraction of each for various tests, as shown in Sections I A, B, and C of the protocol) were divided into two portions each.

One portion of each filtrate was subjected to heating in sealed ampoules and submerged under water at 56°C. for 30 minutes. At the end of this time the tubes were removed from the water bath, cooled quickly in cold water, and the contents were tested for phage content and for their ability to cause the lysis of dead staphylococcus, as indicated in Sections II A, B, and C of Protocol VI.

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[3] This control is essential, for if the filtrate contains live bacteria, the subsequent lysis of dead bacteria, if it occurs, cannot be attributed directly to passage of the active agent, but may be due to the lysis of live bacteria.

Protocol VI.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Culture of Staphylococcus during Progress of Lysis.

Duration of preliminary incubation	A (2 hrs.)					B (4 hrs.)						C (6 hrs.)					
Amount filtered in cc.	Control	50	50	50	50	Control	50	50	50	50	50	Control	50	50	50	50	50
Filtrate in cc.	0	9	9	9	9	0	9	9	9	9	9	0	9	9	9	9	9
Sterile broth in cc.	9	0	0	0	0	9	0	0	0	0	0	9	0	0	0	0	0
Dead staphylococcus suspension in cc.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Incubated at 37°C.

I

Phase titer in cc.		1×10^{-6}					1×10^{-6}						1×10^{-8}				
Turbidity { Immediately	+	0	0	0	0	+	0	0	0	0	0	+	0	0	0	0	1
After 24 hrs.	+	0	0	2	3	+	0	0	2	3	0	+	2	0	1	1–0	0

Portions of each of the fractional filtrates were heated for 30 min. at 56°C. Mixtures were prepared exactly as above with broth and dead bacteria and incubated at 37°C.

II

Phase titer in cc.		1×10^{-6}					1×10^{-6}						1×10^{-6}				
Turbidity { Immediately	+	0	0	0	0	+	0	0	0	0	0	+	0	0	0	0	0
After 24 hrs.	+	0	0	1	0	+	0	0	1	0	0	+	2	0	1	1–0	0

Portions of each of the fractional filtrates were kept at room temperature for 8 days. At the end of this time mixtures were made exactly as above with broth and dead bacteria, and incubated at 37°C.

III

Phase titer in cc.		1×10^{-7}					1×10^{-6}						1×10^{-6}				
Turbidity { Immediately	+	0	0	+	0	+	0	+	0	+	0	+	0	+	0	0	0
After 24 hrs.	+	0	0	+	0	+	0	+	0	+	+	+	+	+	+	0	0

The remaining portion of fractional filtrates was allowed to stand in the room at ±25°C. for 8 days. After this period they were subjected to the test outlined in Sections III, A, B, and C of Protocol VI.

This experiment explains the discrepancy. It appears that when the filter is new and only a small amount of lysed cultures is filtered, only the phage appears in the filtrate, but as more and more of the solution is forced through the same candle, it becomes less selective and allows the passage of the second agent which is capable of causing dissolution of dead bacteria.[4] This is illustrated in the protocol by a diminution of the turbidity of bacterial suspensions in the tubes containing the filtrate, as compared with the turbidity of the controls. In the case in which the lysis in the original mixture was allowed to proceed only 2 hours before filtration (Protocol VI, Section I A), this agent appeared late—only after the passage of the fourth fraction—but as its concentration in the solution increased with the progress of lysis of live bacteria, it appeared in the filtrate sooner and in greater concentration, so that in the last section of the experiment (C) traces of the agent were already present in the second fractional filtrate, and in the fourth and fifth its concentration was sufficient to destroy almost all dead bacteria, as shown by the decrease of turbidity from 4 to 1. Comparison of the results in Sections I, II, and III, on the other hand, indicates that the agent lytic for dead bacteria is present only in freshly prepared filtrates of lysed cultures. If these filtrates are allowed to stand or are subjected to heating, this agent undergoes destruction, while the phage is still present in the solution and is unaffected.[5]

[4] Occasionally, on continued fractional filtration, not only the second active agent but also bacteria appeared in the filtrate, as was indicated by the sterility controls. Such experiments were discarded and repeated, until sterile filtrates were obtained. These findings are of especial interest in connection with the statements in the literature, in which the appearance of growth in the filtrates of lysed cultures is attributed to the existence of a filterable stage in the life cycle of bacteria (13, 14, 26).

[5] While in the experiment recorded in Protocol VI the agent causing the dissolution of dead bacteria seems to be completely destroyed on standing or on exposure to heat at 56°C. for 30 minutes, this is not always the case. Repeating these experiments and employing suspensions of dead bacteria of varying density, we found that this destruction is usually very marked but not complete—with

Failure of Filtrates of a Live Resistant Variant, Grown in the Presence of Bacteriophage, to Induce Lysis of Dead Staphylococcus.

We have shown in the preceding experiments that bacteriophage has no direct lytic action on dead bacteria. Apparently by causing the lysis of susceptible live staphylococcus, it merely sets free a lytic agent preexisting in the bacteria themselves.

If this inference is correct, the incubation of bacteriophage with a live resistant variant instead of with a susceptible staphylococcus should yield a filtrate that will not lyse dead bacteria. Such an experiment was performed with a resistant variant isolated from the susceptible strain of Staphylococcus "G" used previously. It was found that when the experiment was carried out in a manner identical with that shown in Protocol VI, no dissolution of the dead bacteria occurred. On the other hand, dead resistant bacteria were as susceptible to the lytic action of the agent produced by the live susceptible bacteria as were the dead susceptible organisms themselves.

Evidently the failure of the phage to cause the lysis of resistant staphylococcus precluded the liberation of the lytic agent from the latter. However, when susceptible live bacteria were used, the lytic agent was set free and was able to dissolve either the susceptible or resistant dead staphylococci.

In general, the results of the preceding experiment confirm our earlier observations, namely, that the agent responsible for the transmissible lysis of live bacteria is different and independent from that which causes the lysis of dead bacteria. Moreover, the fact that the latter ferment-like agent appears comparatively late during the lysis of live bacteria, that it is thermolabile, and is inactivated on standing,[6] that it is specific in its action, that it does not go through the collodion membrane, that it passes the filters only with difficulty, and causes dissolution of dead bacteria, suggests the possibility that it may be identical with the autolytic endoenzyme which is set free during the lysis of live bacteria.

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very light suspensions of bacteria some residual activity can still be detected, when heavier suspensions of bacteria show no apparent presence of the lytic agent, as will be shown later (Protocol IX).

[6] This inactivation is probably due to its combination with the products of its own activity.

*Analogy between the Agent Dissolving Dead Bacteria and the
Autolytic Enzyme.*

In order to determine whether this ferment-like agent, destroying
dead staphylococcus, can be identified with the autolytic endoenzyme
of this organism, we repeated some of the experiments described above,
using the enzyme obtained from cultures of staphylococcus grown in
the absence of phage.

Protocol VII.

Presence of Specific Bacteriolytic Agent in the Young Cultures of Staphylococcus.

	Dead staphylococci		Dead B. coli		Dead B. pestis caviæ		Dead B. dysenteriæ	
Supernatant fluid from staphylococcus culture in cc....................	5	0	5	0	5	0	5	0
Broth in cc.......................	0	5	0	5	0	5	0	5
Suspension of dead bacteria in cc.....	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Covered with toluene and incubated at 37°C.

Turbidity {	Immediately..........	5	5	5	5	5	5	5	5
	After 5 hrs.*..........	2	5	5	5	5	5	5	5

* The contents of these tubes were tested for the presence of phage after the
completion of the experiment in order to exclude the possibility of lysis having
occurred as the result of accidental introduction of bacteriophage.

The culture of staphylococcus was grown in broth for 18 hours, either aerobi-
cally or anaerobically, the bacteria were removed by centrifuging for 1 hour at
high speed, and 5 cc. of the supernatant fluid were placed in a tube, to which was
added a suspension of dead staphylococcus. Another tube containing 5 cc. of
sterile broth received a similar amount of the same suspension of dead bacteria.
The initial turbidity of the resulting suspensions was recorded by comparison with
the standard turbidity scale.   Chloroform or toluene was then added to the con-
tents of each tube as a preservative, and the tubes were incubated at 37°C.   At
the expiration of 5 hours turbidity was again compared with the standard scale
and changes recorded, as illustrated in Protocol VII.
   In a series of experiments identical with the one just described, suspensions
of dead *B. coli*, *B. pestis caviæ* and *B. dysenteriæ* (Shiga) were subjected
to the action of the supernatant fluid from the 18 hour growth of
staphylococcus, in order to determine whether the action on dead bacteria
was specific, and the results were similarly recorded in terms of turbidity of

the suspensions, immediately after the addition of dead bacteria, and after 5 hours of incubation at 37°C. (Protocol VII).

Thus, in the 18 hour culture of staphylococcus grown in broth in the absence of bacteriophage there was present a specific bacteriolytic agent (presumably enzyme) active against dead staphylococcus. In order to identify more closely this bacteriolytic agent with that which was found to be present in cultures of staphylococcus undergoing lysis by the bacteriophage, we repeated the fractional filtration experiment (see Protocol VI), employing the supernatant fluid of a broth culture of staphylococcus instead of the fluid resulting from the progressive lysis of staphylococcus by the bacteriophage (Protocol VIII). The experiment was carried out exactly as before (Protocol VI) and a detailed description of the procedure has therefore been omitted.

As will be seen, this experiment shows that even after 6 hours of growth the cultures of staphylococcus do not contain enough enzyme in solution for it to be demonstrable by the method used. However, after 18 hours of growth there is a measurable amount of the enzyme present. If these findings are compared with corresponding results in the preceding experiment, it will be observed that in the presence of bacteriophage the enzyme-like substance appears earlier and is present in considerably greater concentration (Protocol VI, Section I), since even the filtrate of 2 hours' growth contains the active agent. This difference might have been expected, since in the presence of phage the rate of growth is more rapid, and also because a number of bacteria are undergoing early lysis, thus setting free the enzyme, whereas in the absence of the phage the enzyme appears in solution, coincident with the late autolysis of bacteria. Moreover, in both cases the first fractional filtrates of the cultures presumably containing the enzyme do not show any activity, and it is only on repeated fractional filtration that the activity becomes demonstrable (compare Protocol VI, Section I A with Protocol VIII, D). Similarly, it was found that exposing to heat the filtrates containing the enzyme, or keeping them for days in the laboratory results in a gradual destruction of the active agent in a manner entirely analogous to that observed in the case of the filtrates of lysed cultures of staphylococcus (Protocol VI, Sections II and III). Here again deterioration of the enzyme

Protocol VIII.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Broth Culture of Staphylococcus.

Duration of preliminary incubation	A (2 hrs.)				B (4 hrs.)				C (6 hrs.)				D (18 hrs.)			
Amount filtered in cc............	0	50	50	50	0	50	50	50	0	50	50	50	0	50	50	50
Amount of filtrate in cc..........	0	9	9	9	0	9	9	9	0	9	9	9	0	9	9	9
Sterile broth in cc...............	9	0	0	0	9	0	0	0	9	0	0	0	9	0	0	0
Dead staphylococcus suspension in cc..	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Incubated at 37°C. and turbidity determined at intervals.																
Immediately.................	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
After 2 hrs.................	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 4 ".................	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 24 ".................	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2–	2–
" 48 ".................	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1–

appeared complete when its activity was tested with heavy suspensions of bacteria, but when lighter suspensions were used, destruction of the enzyme under the conditions of the experiment was found to be incomplete as will be seen in Protocol IX.

So far as we have inquired into the behavior of the unknown agent appearing in cultures of staphylococcus during lysis under the influence of the bacteriophage, and responsible for the dissolution of dead bacteria, it appears in all respects analogous to the autolytic enzyme which can be obtained from older cultures of staphylococcus grown without the phage.

Protocol IX.

The Effect of Density of Bacterial Suspension on the Outcome of the Test of Activity of the Enzyme.

Filtrate of lysed culture kept for 8 days at room temperature in cc......................	5			5			5		
Filtrate of 18 hr. old culture of staphylococcus kept for 8 days at room temperature in cc........................		5			5			5	
Sterile broth in cc............			5			5			5
Suspension of dead staphylococcus in cc..............	0.05	0.05	0.05	0.1	0.1	0.1	0.15	0.15	0.15

Incubated at 37°C.

Turbidity	Immediately......	2	2	2	4	4	4	6	6	6
	After 4 hrs.......	2	2	2	4	4	4	6	6	6
	" 24 "	1−	2−	2	3	4	4	6	6	6
	" 48 "	1−	1	2	2	3	4	6−	6	6

The Effect of the Lysis of Dead Bacteria on the Titer of the Phage.

Our experiments show, we believe, conclusively that the enzyme-like agent is distinct from phage, and its early appearance in the culture is merely incidental to the lysis of living bacteria and in no way connected with the activity of the bacteriophage itself. However, in view of the fact that Gratia and Rhodes (8) report probable regeneration of phage during the lysis of dead bacteria, we investigated this point.

For this purpose, live staphylococci and a corresponding phage were introduced into a flask containing 200 cc. of sterile broth, and incubated at 37°C. for 4 hours. Up to that time the lysis had taken place only partially, and the titer of phage had reached 1×10^{-6} cc., as illustrated in Protocol X. The bulk of the contents of the flask, with the exception of 10 cc., was filtered through a sterile candle in order to thoroughly saturate it, and the filtrate was discarded. The last 10 cc. of the culture were then filtered through the same candle and to this filtrate—presumably containing both the phage and the agent active for dead bacteria—was added a suspension of dead bacteria. The turbidity of the resulting mixture was estimated, a sample was taken out for the immediate titration of the phage content, and the tube was placed in the incubator. After 24 hours of incubation the turbidity and phage content were reestimated.

Protocol X.
Effect of Dead Bacteria on the Phage Titer.

Filtrate in cc	10	0	10
Broth in cc	0	10	0
Suspension of dead bacteria in cc......	0.2	0 2	0

Incubated at 37°C.

	Turbidity	Phage titer	Turbidity	Phage titer	Phage titer
		cc.			*cc.*
Immediately.....................	4	10^{-6}	4	0	10^{-6}
After 24 hrs.....................	3—	10^{-1}	4	0	10^{-6}

The experiment shows that during the partial lysis of dead bacteria there was no increase in the phage. On the contrary, the bulk of the phage disappeared from the solution during the incubation, probably having been adsorbed on dead bacteria.

DISCUSSION.

According to the original conception of d'Hérelle (15), the clearing of bacterial suspensions in the presence of bacteriophage is the result of gradual swelling and eventual bursting of bacteria, due to the accumulation within them of multiplying parasites (*Bacteriophagum intestinale*). Following this bursting, the young parasites are set free in increased numbers to invade other bacteria, and the débris of ruptured bacterial cells is dissolved by the action of lysin secreted by the parasite (16).

Hence, the increase of the concentration of bacteriophage in solution is preceded by the bursting of bacteria, and the destruction of the latter is considered essential for the regeneration of the phage. However, it has been shown repeatedly that the phage titer of a culture shows an increase before the onset of actual lysis of bacteria, and indeed, under certain conditions, it may reach very high concentration without any lysis of susceptible bacteria (17–21). Thus, while lysis of bacteria is the most striking feature of the d'Hérelle phenomenon, it evidently plays no part in the production or regeneration of the bacteriophage, and when it occurs it is secondary to more essential, though obscure changes in bacterial cells which are accompanied by an increase of phage titer. Just what the nature of the process is by which bacteria undergo complete dissolution in the phenomenon of d'Hérelle has not been definitely established. We have shown (22) that there is no valid evidence of the existence of "lysin," as postulated by d'Hérelle, as a secretion of the ultraparasite (16). Moreover, if bacterial débris is dissolved by an enzyme-like lysin secreted by the *Bacteriophagum intestinale*, one would certainly expect that such a lysin would also dissolve dead bacteria, whereas all the experimental data presented thus far indicate that only live and actively growing bacteria are subject to lysis by the phage.

The findings of Twort seemed to us to have offered a means of solving this problem. We have been able to confirm his observations that dead bacteria remain unaffected by the bacteriophage alone, but that they undergo lysis if, in addition to the bacteriophage, homologous live bacteria are present. We have shown in the experiments reported in this paper that bacteriophage itself takes no part in the dissolution of dead bacteria, but acts merely as an incitant for certain changes occurring in live bacteria and leading to their eventual lysis. The dissolution of the dead bacteria takes place at the expense of a lytic enzyme, set free as the result of lysis of the live bacteria.

These findings, taken with our observations on the viscosity of bacterial suspensions in the presence of bacteriophage (23), lead us to infer that the determining factor in the failure of bacteriophage to bring about dissolution of resistant or old bacteria is to be looked for in the failure of these bacteria to swell under the influence of the phage. Apparently the swelling itself (by dilution of intracellular

contents?), or the bursting of live bacteria as the result of the intake
of water, is followed by the dissociation of the intracellular enzyme-
antienzyme complex (24), with consequent activation of the autolytic
enzyme, which attacks not only the débris of ruptured young bacteria,
but if present at the same time, also the dead bacteria.

SUMMARY AND CONCLUSIONS.

We have been able to confirm the observations of Twort as well as
of Gratia, that dead staphylococcus may undergo lysis if, in addition
to a suitable bacteriophage, there is also present live staphylococcus.
Moreover, we have endeavored to ascertain the mechanism of this
phenomenon and have found that in order to elicit it it is necessary to
control the numbers of live and dead bacteria in the mixture. An
excess of dead bacteria interferes with lysis by adsorbing the bacterio-
phage before it has the opportunity to initiate necessary changes in
the live bacteria, so that all lysis is prevented. The phenomenon is
specific, that is, the lysis of live bacteria is accompanied by lysis of
dead bacteria of the same species only. Lysis of dead bacteria occurs
best with staphylococcus, an organism which easily undergoes spon-
taneous autolysis under appropriate conditions. In the case of
B. coli or *B. dysenteriæ* the lysis of the dead bacteria is uncertain.
Dead bacteria need not be present in the mixture at the beginning of
the experiment; they will be dissolved if added any time before, during,
or after the completion of lysis of live bacteria.

If the test is performed so that a suitable semipermeable membrane
is interposed between the dead and live bacteria, the dead bacteria
are not dissolved, in spite of the lysis of live bacteria on the other side
of the membrane. The agent determining the lysis of dead bacteria
is not diffusible, while the principle initiating the lysis of live bacteria
diffuses freely and is demonstrably present on both sides of the mem-
brane. The complete independence of the agent causing dissolution
of dead bacteria from bacteriophage can also be shown by separating
the two agents by means of filtration, or by adsorption on bacteria.

The ferment-like substance responsible for the lysis of dead bacteria
is different from the bacteriophage. It is not diffusible through col-
lodion, it is easily adsorbed on clay filters, it is heat-labile, and is
inactivated on standing.

An agent possessing identical properties was found in cultures of staphylococcus undergoing spontaneous autolysis in the absence of bacteriophage, but in this instance the agent appeared in the filtrates considerably later than it did when phage was present.

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BACTERIUM LEPISEPTICUM INFECTION.

Its Mode of Spread and Control.

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The experiments outlined in this paper were designed to throw some light on the natural mode of spread of *Bact. lepisepticum* infection and to determine a method for its control. The technique employed is based upon the results of a number of previous observations of the spontaneous disease as it occurs in rabbits (1,*a*, 2) and upon measurements of certain properties of host and microbe under limited but rigidly controlled circumstances (1,*b*). It was hoped, therefore, that by eliminating certain variables and submitting certain conceptions to a critical test under natural conditions, it would be possible to ascertain accurately the factors underlying the various phenomena observed.

The test may be divided conveniently into two parts; first, an effort to keep a certain population of rabbits entirely free from *Bact. lepisepticum* infection; second, a study of the behavior of another population of rabbits exposed to the disease in a random and natural manner. To carry out the first part of the experiment, a specially selected group of animals was chosen and placed in a well isolated room, under the care of an experienced attendant; for the second test, the animals were also chosen with care and placed in an isolated room, but were exposed to the risk of infection by a general rabbit caretaker. The details of each test and the results obtained follow.

Spread of Spontaneous Bact. lepisepticum Infection among a Specially Controlled Group of Rabbits.

Materials.—52 rabbits, 3 months old, weighing 800 to 1000 gm. each, were chosen for this experiment. Thirty-six came from a nearby farm which was reported free from cases of clinical snuffles; sixteen were taken from the Rockefeller

Institute breeding room described below. Animals in this breeding room had been entirely free from *Bact. lepisepticum* carriers for 1 year. Hence it was inferred that the young animals employed in this experiment had never been exposed to *Bact. lepisepticum*. The nasal passages of the 52 animals were cultured three times to discover the presence of *Bact. lepisepticum* carriers. None was found.

The rabbits were placed in separate cages measuring about 12 × 12 × 22 inches in dimensions, and distributed uniformly in four stacks of five cages, and one
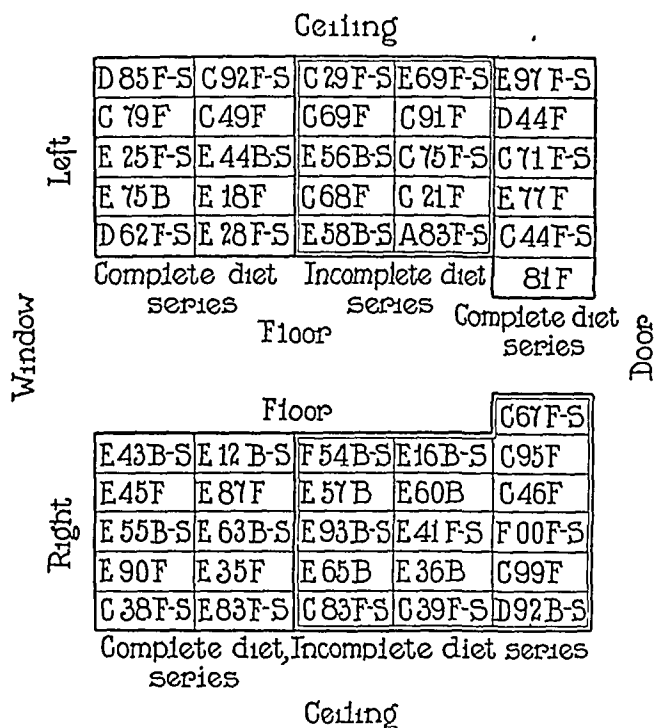
TEXT-FIG. 1. Plan of experimental room showing arrangement and location of animals. F following rabbit numbers = outside farm source; B = breeding room source; -S = depilation, October, 1925.

stack of six, on two sides of a room about 10 feet wide and 20 feet long. A window was located at one end of the room, a door at the other (Text-fig. 1). The temperature was maintained as nearly as possible at 68°, except during one definite interval described below. Two distinct diets were employed—a "complete" ration, consisting of oats, first quality hay, and fresh cabbage or carrot, plus 2 or 3 cc. of cod liver oil daily, and an "incomplete" diet of the oats and hay, plus water without any fresh vegetable or cod liver oil. The animals were cared for by attendants who were also in charge of several hundred other rabbits nearby, many of which suffered from snuffles and carried *Bact. lepisepticum*.

Method.—The group of 52 animals was then submitted to the chances of random infection through the vehicle of the caretakers, the bedding, food, insects, etc. Throughout a period of 2 years as many factors as possible were controlled and analyzed. In order to do this with so small a group, every precaution was taken to keep the experimental variables balanced. Thus, the animals from the breeding room were scattered uniformly among those from the farm. The diet groups were likewise evenly distributed so as to include both series of animals and both sides of the room (Text-fig. 1). The population consisted therefore of a group of animals among which was operating a definite number of controlled and balanced variables.

The animals were kept under strict observation. Notes were made of their general health, the presence or absence of snuffles, and other pathological processes. Nasal cultures were taken at frequent intervals (1, *c*) to determine *Bact. lepisepticum* carriers. All animals dying during this period were autopsied carefully.

After the experiment had been in progress for about 10 months, it was decided to modify atmospheric conditions in the room by arbitrarily altering the temperature and humidity. This was done by turning on the steam heat to full strength in the morning, shutting the doors and windows, and turning on the hot water. The room became filled rapidly with water vapor and reached a temperature of about 90°. At night the heat and hot water were shut off and the window opened wide. This caused the temperature of the room to drop to that prevailing out of doors. These extreme measures were employed from October 22, 1925, until February 4, 1926. At the same time the backs of the animals on the top, middle, and bottom rows on each side of the room were depilated from neck to buttocks by means of sodium sulfide (Text-fig. 1).

On December 1 the surviving animals were given intranasally a 16 hour blood broth culture of the virulent Rivers D strain of *Bact. lepisepticum* (1, *d*). And on December 18 to 23 the entire group was killed and autopsied. Cultures were taken from nasal passages, ears, and lungs.

Results.—The results of these observations are shown in Tables I and III and Text-fig. 2. They will be considered in detail with reference to (*a*) the total population, (*b*) the groups on either side of the room, (*c*) the groups from the farm and breeding room, (*d*) the diet groups, (*e*) seasonal factors, including temperature changes and exposure, and (*f*) individual differences in response on the part of the animals.

(*a*) *Total Population.*—The outstanding events referable to the total population are taken from Table I and Text-fig. 2, and charted in Text-fig. 3. *Bact. lepisepticum* carriers appeared in January, 1925, increased at a more or less uniform rate from 10 per cent to 22 per cent in October. *B. bronchisepticus* carriers rose from 4 per cent in March to 31 per cent in May, and fell to a level of about 25 per cent from

TABLE I.

Summary of Findings on Experimental Group of 52 Rabbits.

Side	Diet	Condition	Dec., 1924	Jan., 1925	Feb.	Mar.	Apr.	May	June	Aug.	Oct.	Nov.	Dec.	Mar., 1926	Apr.	May	July	Sept.	Oct.
Left (26)	Complete (16)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1†	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Carriers *Bact. lep.*	0	1	1	0	3	1	2	1	2	2	—	5	7	9	7	3	7
		Carriers *B. bronch.*	0	1	1	1	1	7	7	6	5	4	—	6	6	6	9	6	4
	Incomplete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1‡	0	1‡	0	0	0
		Snuffles	0	0	0	1	0	0	0	0	0	1	2	0	0	1	0	0	0
		Carriers *Bact. lep.*	0	3	6	3	1	2	4	1	3	4	—	6	4	6	3	2	2
		Carriers *B. bronch.*	0	0	1	1	5	6	4	4	4	3	—	5	3	2	7	5	5
Right (26)	Complete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	1†	0	0	0	0	1*	0
		Snuffles	0	0	0	0	0	0	0	0	0	1	2	3	3	2	2	3	3
		Carriers *Bact. lep.*	0	1	0	1	1	1	1	1	2	3	—	6	7	5	5	4	6
		Carriers *B. bronch.*	1	0	0	0	0	2	2	0	0	1	—	1	1	2	4	3	2
	Incomplete (16)	Deaths	0	0	0	0	0	0	0	1*	0	0	2‡	0	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	1	1	1	1	3	1	1
		Carriers *Bact. lep.*	0	1	1	1	2	3	3	5	4	5	—	9	10	7	10	8	9
		Carriers *B. bronch.*	0	0	0	0	1	1	1	3	5	1	—	4	3	0	2	5	3

* Otitis media: *Bact. lepisepticum.* † Septicemia: *B. coli.* ‡ Pneumonia: septicemia: *Bact. lepisepticum.*
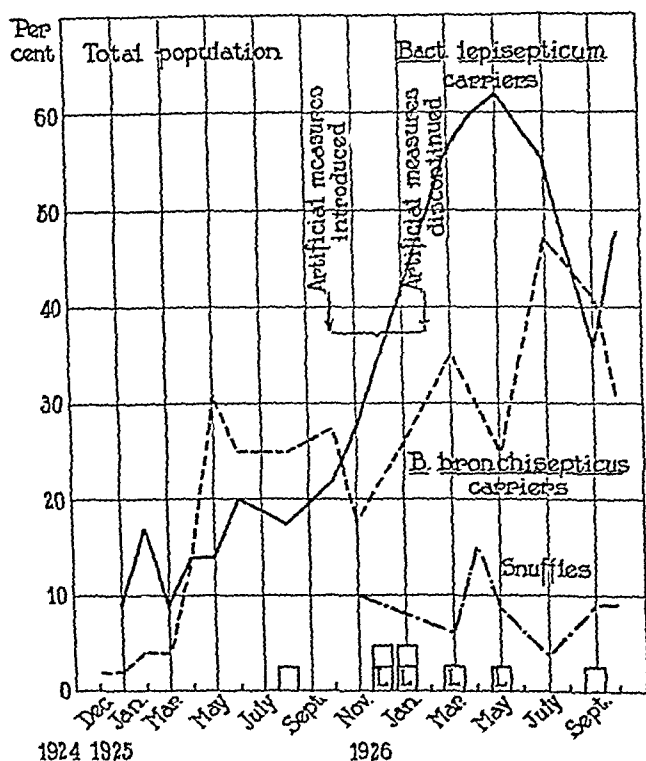
TEXT-FIG. 3.   Reaction of the total population to *Bact. lepisepticum* infection.
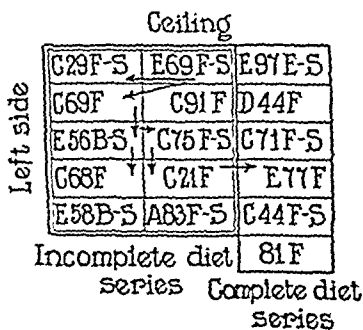
TEXT-FIG. 4.   Cage to cage spread of *Bact. lepisepticum* infection from a single focus, January 30, 1925, to June 25, 1925.

June to October. One death occurred in August. This animal showed bilateral otitis media.

During this period and throughout the 2 years of observation, a spot map was kept, showing the location and spread of *Bact. lepisepticum* and *B. bronchiseplicus* carriers. The early months showed random and scattered foci of *Bact. lepisepticum* infection with a tendency toward local spread. One such focus is charted in Text-fig. 4. The diagram includes the "incomplete" diet series on the left side of
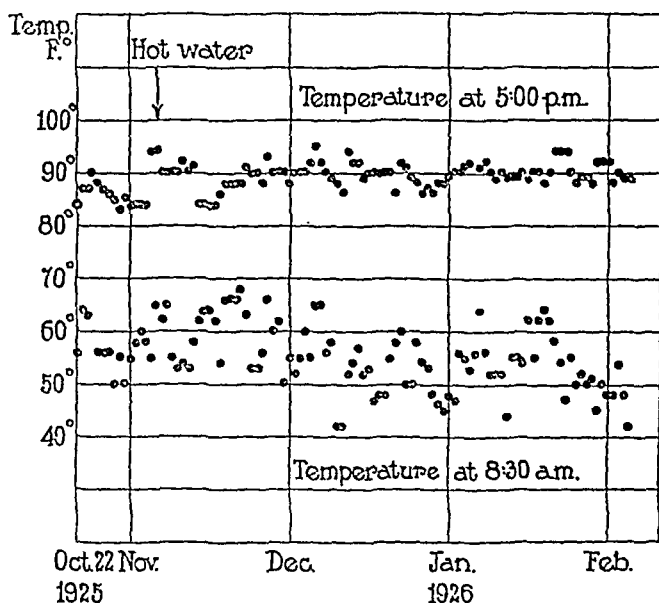
TEXT-FIG. 5. Daily temperature fluctuations in experimental rabbit room.

the room, and six adjacent animals of the "complete" diet series (see Text-fig. 1). The date of appearance of *Bact. lepisepticum* among these animals, recorded in Text-fig. 2, is shown here chronologically by means of arrows.

Apparently the infection started with Rabbit E 69 F-S, and extended to the two adjacent cages, C 29 F-S and C 69 F. From this latter it progressed to E 56 B-S and from there to the three contiguous animals, C 75 F-S, C 68 F, and C 21 F. Finally, it appeared in E 77 F. Evidently, therefore, direct extension of the infection did occur. This possibility appeared the more probable when it was learned that the

attendant always fed the animals in vertical rows, from ceiling to floor. It must be remembered, however, that new foci were appearing throughout the population, and that the attendant was continually passing from the animals in this room to other badly infected stock nearby. Hence, two sources of infection were continually in operation; one, a local spread from established foci in the population, and second, an importation of organisms from without by the attendant.

On October 22, 1925, the artificial temperature fluctuations described above were begun. These procedures continued until February 4, 1926. Daily temperature fluctuations are indicated in Text-fig. 5, in which the 8.30 o'clock morning readings and 5.00 o'clock afternoon readings are recorded. Twice during every 24 hours, therefore, a fluctuation of approximately 35° occurred. Furthermore, on November 6, the hair was removed from the backs of one-half of the entire population (see Text-fig. 1).

These procedures were followed by a striking increase in the percentage of *Bact. lepisepticum* carriers, the appearance of acute and chronic snuffles, and the death of six animals within 7 months. *Bact. lepisepticum* carriers rose to 56 per cent in March, 60 per cent in April, and 62 per cent in May; snuffles appeared and became chronic among about 10 per cent of the population; two animals died in December, two in January, and one in March and May, respectively. Four of the fatalities were due to *Bact. lepisepticum* septicemia or pneumonia. No definite increase of *B. bronchisepticus* carriers was noted during this time. This marked increase in the spread of *Bact. lepisepticum* infection, accompanied by clinical snuffles and a definite mortality, was associated so conspicuously with cold weather and the experimental temperature fluctuations that a causal relation was inferred.

From May to September there was a sharp decline in the percentage of *Bact. lepisepticum* carriers, a decline in the number of cases of clinical snuffles, and in mortality. However, the number of *B. bronchisepticus* carriers increased.

(b) *Left and Right Sides of the Room.*—When the prevalence of *Bact. lepisepticum* infection on the right and left sides of the room is compared (Table I), the same general phenomena are noted. For the first 6 months carriers seemed more abundant on the left side, but during the winter of 1926 the rise was uniform. During the summer

of 1926, however, the carrier rate fell on the left side more rapidly than on the right. Chronic snuffles was more prevalent on the right side, and deaths were about equally distributed.

(*c*) *Breeding Room and Farm Groups.*—The animals from the breeding room appeared to be somewhat more susceptible than those from the farm (Table II). A summary was made at the end of the experiment of the number of deaths, the number of cases of chronic snuffles, of chronic and occasional carriers of *Bact. lepisepticum*, and of animals free of the infection. 25 per cent of the breeding room animals died, as compared with 14 per cent of the farm animals; 31 per cent of the former showed chronic snuffles, 14 per cent of the latter. About the same relative numbers of each group were chronic carriers, but none

TABLE II.

Comparison of Breeding Room and Farm Rabbits and Depilated and Non-Depilated Rabbits.

Group	Died		Chronic snuffles		Chronic carriers		Intermittent carriers		Free of *Bact lepisepticum*	
	No.	Per cent	No.	Per cent	No	Per cent	No.	Per cent	No	Per cent
Breeding room (16)......	4	25	5	31	5	31	2	12	0	—
Farm (36)	5	14	5	14	9	25	12	33	5	14
Non-depilated (22)......	3	13 5	3	13 5	6	27	10	45.5	0	0
Depilated (28)..........	5	18	7	25	7	25	4	14	5	18

of the breeding room animals was free of infection during the entire period; while 14 per cent from the farm never showed *Bact. lepisepticum*.

(*d*) *Diet.*—A comparison of the two diet groups is shown in Table I and Text-figs. 6 and 7. Text-fig. 6 shows, with one exception, that throughout the entire period of observation the percentage of *Bact. lepisepticum* carriers among the "incomplete" diet series was higher than that of the group receiving the "complete" ration. However, there was no striking difference in the percentage of chronic snuffles cases, nor in the mortality rates. Therefore, it is difficult to interpret the effect of omitting fresh vegetables from the rabbits' diet for a period of 2 years, further than to state that no deleterious effect on general health could be observed. However, the fact that these

animals were more prone to carry *Bact. lepisepticum* in their nasal passages is of interest from an epidemiological point of view. Differences in host susceptibility, too slight to be recognized clinically, may apparently be determined by the more delicate bacteriological test.
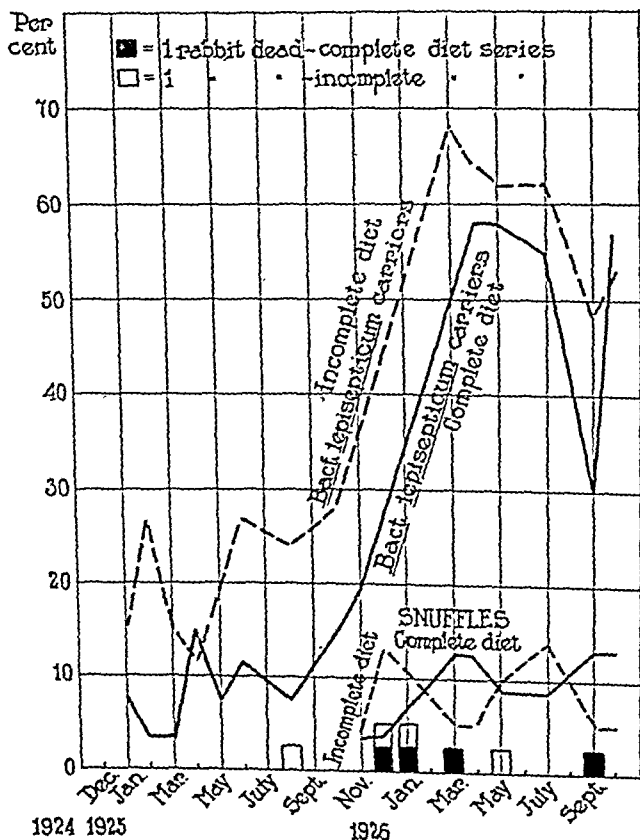
TEXT-FIG. 6. Spread of *Bact. lepisepticum* infection among the "complete" and "incomplete" diet groups.

And this modification of diet, by rendering the individual susceptible to mild infection, whereby he becomes a chronic carrier, may indeed be one influence which, by decreasing host resistance, raises dosage of bacteria available to the population to a dangerously high level.

Text-fig. 7 compares the percentages of *bronchisepticus* carriers in

the "complete" and "incomplete" diet series. No significant differences are apparent.

(e) *Season.*—From the figures available in this experiment, it is difficult to determine any effect of seasonal factors. During the summer of 1925 the percentage of *Bact. lepisepticum* carriers remained moderately low, during the winter of 1926, it rose to a great height,
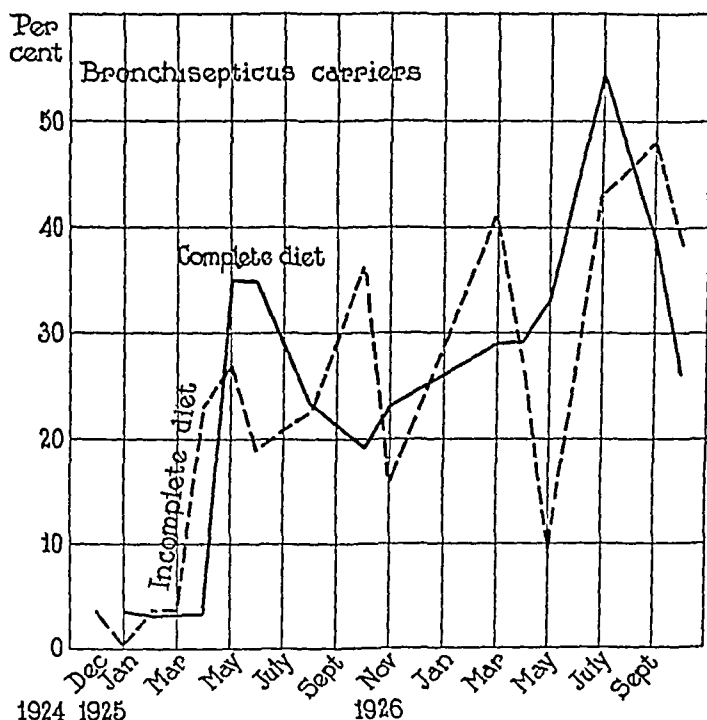
TEXT-FIG. 7. Frequency of *B. bronchisepticus* carriers among the "complete" and "incomplete" diet groups.

and during the following summer declined. However, the complicating factors of artificial temperature changes and depilation make analysis of environmental influences impracticable.

(f) *Depilation.*—The same may be said of the artificial measures of depilation. This was carried out at about the same time that the temperature fluctuations were carried out and was followed by a sharp rise in carrier rate and appearance of chronic snuffles and definite

mortality from *Bact. lepisepticum* septicemia and pneumonia.[1] Probably the three factors, namely, exposure, temperature fluctuation, and seasonal influences were together responsible for the great increase in the prevalence and severity of the disease.

(g) *Behavior of Individual Rabbits.*—Throughout the 2 year period of observation certain animals appeared to be more resistant than others to *Bact. lepisepticum* infection. Hence, at the end of the experiment a final test was employed to emphasize these differences. For this purpose the Rivers D strain of *Bact. lepisepticum* was instilled into the nares of each rabbit. The reaction was slight; two or three acute cases of clinical snuffles developed, but in general the organisms tended to disappear rapidly from the nasal passages (1, *e*). Evidently continual exposure to infection over a period of 2 years had exerted a selective action in weeding out the less resistant individuals, thus leaving a group relatively resistant and showing little difference in response to this organism.

At the end of the experiment the 52 animals were grouped arbitrarily, according to their behavior during the 2 year period (Table III). Those animals that died composed the first group; those that reacted locally with chronic *Bact. lepisepticum* snuffles, the second; those that became chronic carriers of *Bact. lepisepticum*, the third; intermittent carriers, fourth; and finally, those that failed at any time to show *Bact. lepisepticum* in their nasal passages, fifth. Nine animals were placed in the first group. Four of these died from *Bact. lepisepticum* infection and showed in their lungs and hearts' blood the specific organisms. Ten showed symptoms of chronic snuffles and revealed at autopsy an extensive erosion of the nasal turbinates, with accumulations of pus in the sinuses. Nine of them showed pus in one or both middle ears. The mucoid form of *Bact. lepisepticum* was cultured from these lesions. Besides, from the nasal passages of six, ten to twenty colonies of the experimentally introduced Rivers D strain were found. Fourteen animals were chronic carriers. At autopsy two showed pus in the nasal passages and seven unilateral or bilateral otitis media. The mucoid form of *Bact. lepisepticum* was cultured from all of the ear

[1] Four of the six animals dying between December, 1925, and May, 1926, were depilated; on the other hand, a comparison of the reactions of the two groups as a whole (Table II) shows no notable differences.

No.	Fate		Clinical condition	Autopsy findings		
				Nasal passages	Ears	Lungs
E 54 B	Died	8/15/25	Torticollis	Normal	Pus, bilateral	Normal
E 83 F–S	"	12/ 7/25		"	Normal	Congestion, hemorrhage
E 16 B–S	"	12/12/25	Diarrhea; emaciated	Pus	"	Pleuro-pneumonia
D 85 F–S	"	1/ 8/26	Pregnant	"	"	Congestion
E 36 B	"	1/13/26	Snuffles; emaciated	"	"	Congestion consolida-tion
E 56 B–S	"	3/ 8/26	Snuffles; diarrhea	"	"	Hemorrhagic consolida-tion
C 69 F	"	5/21/26	Good	No pus	"	Hemorrhagic
E 90 F	"	9/16/26	Emaciation; torticollis	" "	Pus, bilateral	Normal
C 37 F–S	"	11/25/26	Cellulitis	" "	No pus	"
E 55 B–S	Killed	12/18/26	Emaciated; snuffles	Erosion; pus	Pus bilateral,	"
E 43 B–S	"	"	Good	" "	" "	"
C 38 F–S	"	"	"	Normal	Normal	"
E 57 B	"	"	"	Erosion; pus	Pus, bilateral	"
E 93 B–S	"	"	"	" "	" left	"
C 30 F–S	"	"	"	" "	" bilateral	"
C 95 F	"	"	"	" "	" left	"
A 85 F–S	"	"	"	" "	" right	"
E 63 B–S	"	"	"	" "	" bilateral	"
E 87 F	"	"	"	" "	" "	"
81 F	"	12/20/26	"	Pus	" "	"
E 60 B	"	"	"	"	Normal	"
E 18 F	"	"	"	Normal	Pus bilateral	"
C 68 F	"	"	"	"	" "	"
E 58 B–S	"	"	"	"	" right	"
E 41 F–S	"	"	"	"	" left	"
D 92 B–S	"	"	"	"	" "	"
C 83 F–S	"	"	"	"	" "	"
F 00 F–S	"	"	"	"	Normal	"
E 44 B–S	"	"	"	"	"	"
E 12 B–S	"	"	"	"	"	"

III.

Experimental Rabbits.

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
Sterile	No *Bact. lep.*	*B. coli*	Sterile	Deaths
B. coli	" " "	Sterile	*B. coli*	"
Bact. lep. M	*Bact. lep.* M	"	*Bact. lep.* M	"
B. coli	" " "	"	*B. coli*	"
Sterile	*B. bronch. Bact. lep.* M	"	*Bact. lep.* M	"
Bact. lep. M	*Bact. lep.* M	"	" " "	"
" " "	*B. bronch. Bact. lep.* M	"	" " "	"
Sterile	No *Bact. lep.* M	—	—	"
"	*B. bronch.*	—	—	"
—	*Bact. lep.* D ++ M Inf.	*Bact. lep.* M	—	Chronic snuffles
—	" " " ++ " ",	" " "	—	" "
—	" " " ++	—	—	" "
—	*B. bronch.* Inf. *Bact. lep.* M Inf.	*Bact. lep.* M	—	" "
—	" "	" " "	—	" "
—	*Bact. lep.* D ++ M Inf.	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " M	" " "	—	" "
—	" " "	" " "	—	" "
—	" " "	" " "	—	Chronic carriers
—	" " "	—	—	" "
—	" " D	*Bact. lep.* M	—	" "
—	" " "	" " "	—	" "
—	" " " + M Inf.	" " "	—	" "
—	" " " Inf.*bronch.*++	" " "	—	" "
—	*Bact. lep.* D+	" " "	—	" "
—	" " " +	" " "	—	" "
—	" " " G+	—	—	" "
—	" " D+++	—	—	" "
—	" " " ++	*Bact. lep.* M	—	" "

TABLE III

No.	Fate		Clinical condition	Autopsy findings		
				Nasal passages	Ears	Lungs
C 99 F	Killed 12/21/26		Good	Normal	Normal	Normal
C 49 F	"	"	"	"	"	"
C 79 F	"	"	"	"	"	"
E 65 B	"	12/22/26	"	Pus	"	"
C 44 F–S	"	"	"	"	Pus, left	"
C 21 F	"	"	"	"	Normal	"
C 91 F	"	"	"	Normal	Pus, bilateral	"
D 62 F–S	"	"	"	"	" left	"
E 69 F–S	"	"	"	"	" "	"
E 77 F	"	"	"	"	" "	"
C 46 F	"	"	"	"	Normal	"
C 75 F–S	"	"	"	"	"	"
D 44 F	"	"	"	"	"	"
E 45 F	"	"	"	"	"	"
E 75 B	"	"	"	"	"	"
E 35 F	"	"	"	. "	"	"
E 28 F	Reserved		"	—	—	—
C 67 F–S	Killed 12/23/26		"	Normal	Pus, bilateral	Normal
C 92 F–S	"	"	"	"	Normal	"
C 29 F–S	"	"	"	"	"	"
E 97 F–S	"	"	"	"	"	"
E 25 F–S	Reserved		"	—	—	—

Bact. lep. D = Rivers D type, instilled artificially 12/1/26.
Bact. lep. M = Mucoid type, spontaneous infection.
Bact. lep. G = Variant type, from Rivers D.
Inf. = Many colonies.

—Concluded.

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
—	*Bact. lep.* D+++	—	—	Chronic carriers
—	" " " ++ G++	—	—	" "
—	" " M	—	—	" "
—	" " D+	—	—	Intermittent carriers
—	" " M Inf.	*Bact. lep.* M	—	"
—	" " D Inf.	—	—	"
—	*B. bronch.*	*Bact. lep.* M	—	"
—	D + *B. bronch.* Inf.	" " "	—	"
—	"++	D Inf.	—	"
—	*B. bronch.*	*Bact. lep.* M	—	"
—	*B.bronch.*Inf.*Bact.lep.* D+	—	—	"
—	*Bact. lep.* D+	—	—	"
—	*B.bronch.*Inf.*Bact.lep.* D+	—	—	"
—	*Bact. lep.* D Inf.	—	—	"
—	" " " "	—	—	"
—	*B. bronch.* Inf.	—	—	"
—	—	—	—	"
—	*Bact. lep.* D++	*Bact. lep.* M	—	Free
—	" " Inf.	—	—	"
—	*B.bronch.*Inf.*Bact.lep.* D+	—	—	"
—	" " " +	—	—	"
—	—	—	—	"

lesions and from the nasal passages of four. The Rivers D strain of *Bact. lepisepticum* was cultured from the nasal passages of eleven. Fourteen of the animals were classed as intermittent carriers. At autopsy pus was found in the nasal passages of three and in the middle ears of five. The mucoid form of *Bact. lepisepticum* was recovered from the ears of three; the D form from one. From the nasal passages of one mucoid colonies of *Bact. lepisepticum* were obtained; from nine, D forms. Five animals showed no *Bact. lepisepticum* in their nasal passages at any time during the experiment. At autopsy the nasal passages of all appeared normal. Pus was found in the middle ear of one. From here the mucoid form of *Bact. lepisepticum* was cultured. All four showed a few colonies of the Rivers D form.

When the animals are placed in these arbitrary groups, their differences in behavior appear the more striking. That some animals remained clinically healthy and free of infecting organisms during summer and winter, and at the time when temperature fluctuations and exposure were extreme, cannot be explained by chance. They were scattered amongst their companions, many of which were carriers, some snuffles cases, and still others ill with pneumonia. It seems more reasonable to regard the differences in behavior of these animals as due to innate and non-specific differences in their resistance.

Virulence.—At various times during the experiment, virulence titrations were made on strains of *Bact. lepisepticum* recovered from the nasal passages of various individual rabbits in the room. The technique of these tests has been discussed elsewhere, and the results of a number of them recorded in detail. The critical titrations were made by instilling equal doses of the various cultures into the nasal passages of young rabbits of similar age and weight, obtained from the breeding room. Subsequently, with these results as a basis, the mouse titration method was employed. This technique, although far more artificial, and of little or no value unless controlled by "natural" virulence titration, enabled us to examine a greater number of cultures and at more frequent intervals.

Many of these tests have been described previously.[2] In one instance[3] the results of intranasal titrations in rabbits of Cultures 544

[2] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362, 366–376.

[3] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362.

from Rabbit E 44 and 329 from Rabbit C 29 are summarized.   In Tables IV, V, and VI the results of the intraperitoneal mouse titrations are shown.   Culture 590 came from Rabbit E 90; Culture 560 was obtained on three different occasions from Rabbit E 60.   Thus the strains in these tables may be identified: those in the 500's came from E rabbits; in the 400's from D rabbits; in the 300's from C rabbits, etc.   These titrations emphasize two facts; first, that the form of *Bact. lepisepticum* brought into the room by the attendant and becoming endemic in the population was of the mucoid variety, similar to that found at the rabbit farm in New City, at Saranac, New York, and in other rabbits at the Rockefeller Institute; and secondly, that the virulence of all of these cultures proved equal and of moderate degree.

General Considerations.—The analysis of the part played by certain factors in the spread of *Bact. lepisepticum* throughout this population is rendered easier by reason of the control of disturbing influences. All the animals were exposed equally to the chance of infection so that theoretically those in the "complete" diet group should have been slightly more resistant than those receiving the "incomplete" ration.   We assume that none of the rabbits had been previously exposed to the infection, and that none of the infecting organisms was in the room at the beginning of the experiment.   We know that the infecting potentialities or virulence of the various strains of *Bact. lepisepticum* remained uniform throughout the entire period, and that the source of infection—attendants carrying organisms from infected rabbits in the neighboring room—remained constant.

At the outset, foci of infection were established in the nasal passages or middle ears of the rabbits naturally more susceptible, or rendered so by dietary deficiencies.   During the succeeding 6 months available dosage was maintained at a level by chance contact with the attendant and by spread from local foci.   Throughout the winter of 1925–26, procedures similar but more drastic than natural conditions were carried out to decrease further the resistance of the population. Dosage (carrier rate) increased rapidly, the weaker individuals succumbed to *Bact. lepisepticum* pneumonia and septicemia, and others developed local otitis media and snuffles.   Still others, exposed to infection, remained refractory.   As summer came on and the tem-

perature fluctuations were less severe, the population, reduced in numbers by the selective weeding our process, became more resistant, dosage fell, and cases of snuffles and numbers of deaths became less frequent.

In this experiment, therefore, factors influencing host susceptibility influenced the dosage of available bacteria and thereby determined the amount and severity of infection ensuing.

Control of Bact. lepisepticum Infection. Description of a Successfully Operated Rabbit Breeding Room.

As stated in the introduction, the purpose of this experiment was to test the conclusions derived from our studies of rabbit snuffles and pneumonia by attempting to keep a population of rabbits free of *Bact. lepisepticum* infection. The various procedures in this experiment were governed by two principles: first, the maintenance of host resistance at a maximum, and second, the decreasing of available dosage of *Bact. lepisepticum* to a minimum.

A special room was chosen in the midst of the space used for our normal rabbit stock. The room measures about 15×30 feet and has accommodations for thirty breeding females, ten males, and about fifty weaned young stock. Before bringing any animals into the room, the walls and floors were scraped carefully and washed with lysol, and the breeding cages were sterilized. The rabbits employed were selected from the normal stock obtained from various dealers within a radius of 200 miles of New York City. Such animals, upon their arrival at the Institute, were placed in isolation rooms, where they were observed for a period of 2 to 4 weeks. During this period any animals showing clinical evidence of snuffles were discarded.

During the spring and summer of 1923 several hundred of these animals were cultured from the nasal passages to detect the presence of *Bact. lepisepticum*. In view of the usual high percentage of carriers (1, *f*), a great many animals were examined before a suitable stock of breeders was obtained. No animal was admitted to the breeding room until three successive nasal cultures failed to demonstrate the presence of a single colony of *Bact. lepisepticum*. When an animal was finally admitted, its fur was sponged carefully with 5 per cent lysol. Thus by September a stock of about thirty females and ten males was accumulated.

The animals were cared for by an experienced attendant who has maintained a strict régime of cleanliness. In general, the door to the room is kept locked. Before entering the room, the attendant is careful to change his coat and wash his hands. The food is always taken from a fresh supply, and the animals are given a considerable amount of personal attention. The temperature in the room is regulated carefully at about 68°. The cages are cleaned every 2nd day.

The results of this experiment are striking, for by continuing the above careful methods of supervision we have maintained a breeding room entirely free of *Bact. lepisepticum* infection.[4]

At monthly intervals from September, 1923, to March, 1924, the entire breeding room was examined bacteriologically. Again, on September 24, 29, October 6, and December 17, each animal was cultured from the nasal passages. On March 18, 1925, twenty-two showed organisms of the *B. coli* group on hemolyzed blood agar plates streaked from cultures of the nasal passages. These organisms we have always considered as intestinal contaminants, occurring only at the external nares. Twelve showed *B. bronchisepticus*. These animals continued to be consistent carriers of this organism from the time they were selected for the breeding room until they died, or were discarded. None showed colonies of *Bact. lepisepticum*. On several occasions during 1926 young stock taken from this room have been cultured. On no occasion has *Bact. lepisepticum* been recovered. There have been no cases of pneumonia in the room, no clinical snuffles, no abscesses, and no otitis media.

The breeding record is summarized in Table IV. In it are tabulated: (1) the number of young weaned 2 months after birth; (2) the month of these weanings; (3) the number and date of sterile matings; (4) the date and number of times the young were destroyed; and (5) the fate of the various breeders. The males are not included in this

[4] A few of the animals are affected with ear canker. This is treated in its early stages by local applications of tincture of iodine and lanolin. Likewise there is a certain amount of pinworm infection present, which can only be demonstrated at autopsy.

In 1924 some of the animals showed at autopsy the pathological changes characteristic of rabbit encephalitis. Clinically, the animals appeared perfectly healthy. No further diseases have been found.

TABLE

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.	May
1					3				4			5							7		
2			6			2			M.										4		
3											6						4				
4			4					3	D.												
5			x					2		4				4			6		M.		
6								2 M.	D.												
7				4				3						5					3	D.	
8					2							5			M.			x	M.		D.
9								3	D												
10										4					3				5	Dis	
11										5					x					4	
12										3					x				2		
13																4		x		x	
14													4	M.				2		M.	
15																3		D.			
16																		5		x	
17																			3		
18																5			1		
19																		6	D.		
20															6				2:x		
21															3			1			
22														x	x	M.			M.		
23															3	x					
24															7				7		
25															x				2	Dis.	
26																			4		
27																		1		x	
28																x					
29																		1			
30																.	M.				
31															x					4	
32																	2				
33																		1			

D., signifies died; x, destroyed; Dis., discarded; M., sterile mating.

IV.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1927	Total
4				6	D.															29
	2	M.		Dis.																14
5							4	Dis.												19
																				7
	6			1	1				5					3	Dis.					32
																				2
																				15
																				7
																				3
																				12
		2	Dis.																	11
		1		1	Dis.															7
		3			x	Dis.														7
		1			Dis.															7
																				3
				2			3		M.				5	Dis.						15
			3		M.		M.	4					M.	M.	Dis.					10
		1					4			3		4			3					21
																				6
			3	D.																11
		6	x				8						5	Dis.						23
		1			2	Dis.														3
	2				2						5		4				3			19
3				5	M.				7	x					M.	Dis.				29
																				2
3	M.			4					6				7		M.	M.	M.	M.		24
	M.			2	D.															3
2	M.	x		2	M.				6					2			4			16
4				5					8				1		M.			3		22
	r	Dis.																		4
																				4
	1x			1				6	Dis.											10
			5					8												20

TABLE IV—

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.	May
34							6														
35							D.														
36							M.														
37																					6
38																					6
39																					1
40							x														
41																				1	
42							7														
43																					
44																					
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46																					
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59																					
60																					
61																					
62																					
63																					
64																					
Total....			10	4	5	2	0	10	7	16	6	10	0	13	22	4	18	15	48	14	17

Continued.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1927	Total
3	x				5															
	x				5					4		5								
4	M.	D.								4										
	M.		2								3			M.	M.	Dis.				28
				5		5	M.			6				M.	D.					12
	2		x		4			7			M.			M.						4
	5			4		M.				7		M.	M.	M.						19
	1	D.		4					4	1			4	M.						25
2			5	Dis.									2	M.	M.					12
x		M.	M.		7															15
x		x		7					2											2
	x	M.	4				3			M.	M.			M.						14
				7	M.	Dis.	3		M.		3		M.	M.						9
			7			1							5							13
		4	M.		2	D.		M.		M.	Dis.									17
		3																		7
	x	3			2		2		x	M.	Dis.									8
					7		2			3										6
		4	M.				5	M.	M.	M.										5
	5			M.			x			1		x								10
			2M.							6										12
		2			3						2									5
		2			M.	M.					7									11
		1			1			M.		6										2
		4			4		M.				2									9
					2															5
					4		•		4											2
					2				2											4
																				2
										4										4
										5										4
								2												5
										4										6
										4										4
38	13	19	6	26	33	18	41	42	15	45	33	32	21	10	28	15	19	23		701

table. The deaths recorded were due to parturition complications. None of these animals was infected with any microorganism. Animals were discarded for several reasons: first, for sterility or neglect of young, and secondly, for not conforming to the desired type. Dutch, Belgian, and albino crosses were specially selected because of their tendency to produce rapidly developing, small, heavy stock. During the year 1924, 95 young were weaned, an average of 8 per month and 3 per rabbit. In 1926, 265 were weaned, 22.2 per month, 9 per rabbit, and in 1926, 327 were weaned, 27.2 per month, 11 per rabbit.

The results of this experiment indicate that it is possible to maintain a stock of rabbits free from *Bact. lepisepticum* infection, although surrounded by groups in which the disease is widespread, by proper attention to the general condition or "natural" resistance of the animals, and by measures which minimize the available dosage of pathogenic microorganisms.

DISCUSSION AND SUMMARY.

In this paper we have attempted to describe the manner of spread of an endemic, native, respiratory infection and a method for its control. The essential factor determining the prevalence of such an endemic disease is, we believe, host susceptibility, which is controlled by hereditary and environmental influences. Furthermore, it seems probable that the amount of this population susceptibility determines the dosage of specific microbes available to the population.

An increase in dosage in the herd is followed by an increase in the spread and severity of the infection, and a decrease by a corresponding alleviation. Hence, two methods for the prevention of epidemics are available: (1) an enhancement of population resistance, and (2) the reduction to a minimum of available dosage. These procedures have proved successful for 3 years in maintaining a population of breeding rabbits, in the midst of a badly infected community, entirely free from *Bact. lepisepticum* infection.

Confirmation of the above conclusions has been gained from other studies in the field of experimental epidemiology. Dr. D. T. Smith (2), at Saranac, New York, found that changes in population susceptibility were responsible for a severe outbreak of *Bact. lepisepticum* infection and septicemia. Freund (3), at Berlin, has just published

an interesting account of respiratory epidemics of rabbits and guinea pigs, apparently brought about by sudden changes in temperature and housing conditions. Pneumonia and Pasteurella infection, endemic in the population, increased suddenly in extent and severity. Nevertheless, neither endemic nor epidemic strains of the microorganisms were found to be especially virulent. Dr. Theobald Smith (4), in a study of paratyphoid epidemics of guinea pigs, has made similar observations. He noted that pregnant females acted as the foci of infection, and that from these individuals, presumably of lessened resistance, the bacteria were given off and infection was spread.

The studies in experimental epidemiology are rapidly reaching a stage where they may be applied to the problems of human disease. Indeed, more recent observations of the mode of spread of pneumonia (5–7), scarlet fever (8), typhoid (9, 10), plague (11), diphtheria (12–14), measles (15), and tuberculosis (16–18) increasingly show a tendency to discard the theory of fluctuating microbic virulence and to emphasize the importance of the host factors.

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STUDIES ON BACTERIAL ENZYMES.

IV. The Maltase and Lipase of the Botulinus Bacillus.

By JAMES M. NEILL, Ph.D., and WILLIAM L. FLEMING.

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

INTRODUCTION.

The present paper reports a study of the maltase and lipase derived from *botulinus* bacilli; the activity of the enzyme was demonstrated with sterile solutions of the bacterial cells not only devoid of living bacteria but freed from cell fragments by Berkefeld filtration. The investigation was undertaken as a step toward obtaining further knowledge of the mechanism of metabolism of this toxin-forming species of bacterium. The fact that the *botulinus* bacillus is an anaerobe lends more general interest to a study of its enzymes since relatively little work has been done on the enzymes of anaerobic organisms.

Although maltase and lipase derived from different bacteria have been reported by a number of workers, no previous demonstration of these enzymes from *botulinus* bacilli appear in the literature. Several reports (1) have been made of the proteolytic action of sterile fluids obtained from broth cultures of *botulinus* bacilli; this work has been done with supernatant fluids of broth cultures. The sterile bacterial solutions used in the present study differ from the preparations of previous workers in that they contain the intracellular substances liberated when the bacterial cells are disintegrated in addition to the soluble products of growth.

EXPERIMENTAL.

Methods.

Preparation of Bacterial Extracts, or Enzyme Solutions.—The bacterial extract, or enzyme solution, was prepared from a representative, toxin-producing strain of *botulinus* bacilli (Type A) furnished by Dr. Jacques Bronfenbrenner of The Rockefeller Institute for Medical Research.

Large Florence flasks were filled aseptically with sterile meat infusion broth,

placed in the Arnold sterilizer, and steamed for 1 hour to drive out dissolved air; a heavy layer of sterile vaseline was then poured over the hot broth; the culture medium extended into the neck of the flasks so that only a small surface of the fluid required the vaseline seal for protection from air.   This air-free medium was inoculated with a young culture of *botulinus* bacilli and incubated at 35°C. for 15 hours.   The broth culture was then distributed into sterile, 250 cc. centrifuge bottles, sealed with vaseline, and centrifuged.   The supernatant was removed and the sedimented bacilli from each 250 cc. bottle were suspended in about 5 cc. of the supernatant fluid.   (The bacilli were suspended in the supernatant culture fluid because we believe that the presence of certain constituents of the broth either facilitates the subsequent dissolution of the bacteria or renders the liberated bacterial substances more soluble.)

The bacterial suspension was placed in long, narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus (2) and meningococcus (3) extracts.   The *botulinus* bacilli do not disintegrate easily as do pneumococci and meningococci and it was necessary to subject the suspension to about 100 repeated freezings and thawings.   After this treatment, microscopic examination of the *botulinus* suspension indicated that the bacilli were well broken up, since the prepared films consisted almost entirely of bacterial detritus with only an occasional, definitely outlined bacillus.   The suspension of disintegrated bacilli was centrifuged at high speed for 1 hour and the supernatant fluid was filtered through a Berkefeld candle.   The filtered solution was distributed into long narrow test-tubes, sealed with vaseline, and stored at 2°C.

The sterile filtrate obtained represents an extract of the intracellular substances liberated when *botulinus* bacilli are disintegrated by repeated freezing and thawing.   Since the bacilli were originally suspended in small portions of the supernatant culture fluid, these extracts contain broth and the soluble products liberated into the culture fluid during early growth of the culture, in addition to the intracellular substances derived by dissolution of the bacteria.   After Berkefeld filtration, the extracts of the *botulinus* bacilli are perfectly clear and are indistinguishable in appearance from uninoculated broth.   The presence of a considerable amount of dissolved protein material derived from the disintegrated bacterial cells is indicated by the heavy protein precipitation which occurs when these clear solutions are heated.

These sterile extracts of *botulinus* bacilli should contain both the exocellular and endocellular enzymes of the *botulinus* bacillus, provided that the enzymes are neither destroyed during the preparation processes nor held back by the Berkefeld filter.   The term "enzyme solution," when used in the following text, refers to the bacterial extracts described above.

Sterility Controls.—After filtration, the sterility of the enzyme solution itself was proved by cultures in glucose broth.   No antiseptics were added to any of the hydrolysis mixtures, since the sterility of each mixture was controlled by cultural methods.

Demonstration of the Hydrolysis of Maltose by Botulinus Enzymes.

The carbohydrate-fermenting proclivities of *botulinus* bacilli are not pronounced. While glucose is commonly fermented, there is considerable dispute in the literature concerning their fermentation of the common disaccharides, due largely to the fact that different strains of *botulinus* vary in their disaccharide fermentation. Living cultures of the strain used in this investigation fermented maltose rather slowly, and seemed completely devoid of the property of producing either acid or gas from lactose or sucrose. These fermentation properties, while not agreeing with all reports in the literature, are possessed by perhaps the majority of strains of *botulinus* (Type A), since the order of frequency of fermentation of these sugars by different strains seems to be maltose, sucrose, and lactose (4).

Since the carbohydrate-splitting enzymes of *botulinus* bacilli had never been demonstrated, it seemed of interest to determine whether or not the sterile extract prepared from the intracellular substances of the bacilli possessed the property of hydrolyzing maltose. Tests were included with sucrose and lactose as representing disaccharides not fermented by the living cultures of this strain, as well as with maltose . which is slowly fermented by the living culture.

Hydrolysis Mixtures.—Sterile solutions of maltose, sucrose, and lactose were prepared as follows: 30 per cent solutions of each of the sugars were boiled for 15 minutes and then added aseptically to sterile 0.1 M phosphate (pH 6.9) in amount sufficient to give a final concentration of 3.0 per cent of the sugar. The final solutions were distributed into sterile Pyrex tubes.

The hydrolysis test mixtures were prepared by adding 2.0 cc. of the *botulinus* enzyme solution to one tube of each of the three sugar solutions. (This relatively large amount of enzyme was used because preliminary experiments had shown that the maltase action of the *botulinus* extract was weak and difficult to demonstrate.) One portion of the enzyme solution was boiled for 15 minutes to inactivate the enzymes, and a series of controls of heat-inactivated enzyme was prepared for each of the substrates.

The mixtures of enzyme solution plus the sugar substrate were incubated for 72 hours and then tested for the presence of hexoses.

Detection of the Hexose Products of the Enzyme Action.—The hydrolysis of the disaccharides was tested by the biological method described in detail in preceding papers (3, 5). The method is based upon the acid fermentation of the hexoses by bacteria which do not attack the disaccharide from which the hexoses are derived. 1 cc. of each of the test and control hydrolysis mixtures was put into a sterile Pyrex test-tube; 1 cc. of a suspension of bacteria which produce acid from hexoses but which do not attack the test disaccharide was then added to the different tubes. (An atypical strain of colon bacilli which does not attack maltose was added to the

maltose tests; Shiga dysentery bacilli which do not attack maltose, lactose, or sucrose were used for the lactose and sucrose tests was well as for the maltose series.)

These "fermentation mixtures" (equal portions of hydrolysis test mixture plus the bacterial fermenting agent) were shaken, incubated in the water bath at 38°C. for 1 hour, and then centrifuged at high speed to remove the bacteria.   The production of acid in the "fermentation mixtures" was detected by colorimetric estimations of the pH of the clear supernatant fluids of each of the test mixtures. The series of controls consisting of heat-inactivated enzyme plus the test disaccharide serve to limit all changes in reaction of the fermentation mixtures to acid produced by the fermentation of hexoses previously formed by the active *botulinus* enzyme.

The protocol of the experiment is summarized in Table I.

TABLE I.

Action of Botulinus Enzyme Solution upon Maltose.

Hydrolysis mixture		pH after action of bacteria used as fermenting agent	Change in pH due to bacterial fermentation of the hexose previously formed by *botulinus* enzyme
		pH	ΔpH[1]
Maltose	Active *botulinus* enzyme solution	7.0	0.6
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Sucrose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Lactose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0

[1]ΔpH when used in Tables I to IV indicates change (decrease) in pH.

From the results of this experiment (Table I), it is evident that the *botulinus* enzyme solution contains an active maltase, which can be rendered inactive by heat.   The maltose-splitting activity of the solution of the intracellular substances of the *botulinus* bacilli is relatively weak in comparison to the activity of the same type of bacterial solution prepared from either meningococci (3) or pneumococci (5). This relatively weak action of the sterile solution of the *botulinus* bacilli is paralleled by the slow fermentation of maltose by living cultures of the strain of *botulinus* from which the enzyme solution itself was prepared.

It is also evident in Table I that no acid is formed by the hexose-fermenting bacteria when added to previously incubated mixtures of the *botulinus* enzyme and lactose or sucrose solution.   This fact may be accepted as proof of the absence of lactase or sucrase in the sterile extract of the *botulinus* bacilli.   The failure of the sterile extract of the *botulinus* bacilli to hydrolyze either lactose or sucrose is in agreement with the inability of living cultures of the same strain to produce either acid or gas from the same disaccharides.   Apparently,

TABLE II.

Splitting of Tributyrin by Sterile Extracts of Botulinus Bacilli.

Test mixture	Amount of bacterial extract	Reaction of test mixtures after 48 hrs. at 38°C.	Change in reaction due to splitting of tributyrin
	cc.	*pH*	*ΔpH*
Tributyrin plus active bacterial extract	0.8	6.3	1.2
	0.2	6.8	0.8
Tributyrin plus heat-inactivated bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus active bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus heat-inactivated bacterial extract	0.8	7.6	0
	0.2	7.7	0
Tributyrin solution plus broth	0.8	7.5	0
	0.2	7.6	0

the general rule (5) that the acid fermentation of disaccharides depends upon their preliminary hydrolysis to hexoses holds true for the anaerobic *botulinus* bacillus.

Demonstration of Lipase Action of Botulinus Enzyme Solutions.

The lipase action of the *botulinus* enzyme solution was demonstrated by tests of the production of acid from the splitting of tributyrin.

A 2 per cent solution of tributyrin prepared in 0.1 M phosphate (pH 7.8) was sterilized at 120°C. and 5.0 cc. portions of the sterile solution distributed into sterile tubes; 0.8 cc. and 0.2 cc. of the enzyme solution were then added to separate

tubes of the tributyrin. Three series of controls were included to limit the pro-
duction of acid to that formed through the action of the enzyme on the tributyrin;
(1) phosphate solution plus unheated enzyme; (2) phosphate solution plus heated
enzyme; (3) tributyrin solution plus broth. The first two series served as controls
on the possibility of changes in reaction due to changes in the enzyme solution it-
self; the third series controlled the possibility of a spontaneous hydrolysis of the
tributyrin itself during the incubation period allowed for enzyme action. A
fourth control series consisting of heat-inactivated enzyme plus tributyrin solu-
tion was included to prove that the lipase was a heat-labile substance.

The hydrolysis test mixtures and the series of controls were incubated at 38°C.
for 48 hours. Tests for the splitting of the tributyrin were made by determining
the changes in reaction of the various mixtures. The results are given in Table II.

The results presented in Table II furnish evidence of the action of
lipase derived from *botulinus* bacilli. There is a direct relation be-
tween the extent of the hydrolysis and the concentration of the
sterile bacterial extract.

Resistance of Botulinus Lipase to Exposure to Air.

The lipase of Pneumococcus is susceptible to oxidation and can be
inactivated by treatment with an oxidizing agent, such as hydrogen
peroxide (6), but it is considerably more resistant to oxidizing agents
than are the carbohydrate-splitting enzymes of the same bacteria.
Since the *botulinus* bacillus is an anaerobic organism, it seemed of
particular interest to determine the effect of exposure to air upon the
activity of its lipase enzyme.

The question of the effect of exposure to air was investigated from
two points of view: (1) the effect of exposing the bacterial extract to
air before its addition to the tributyrin solution; (2) the effect of free
exposure of the mixture of tributyrin and bacterial extract during the
incubation period allowed for the enzyme action.

The effect of previous aeration of the bacterial extract upon its subsequent
lipase activity was studied as follows: 5.0 cc. of the sterile bacterial extract were
placed in a shallow layer in a 300 cc. Erlenmeyer flask; another portion of the
same bacterial extract was placed in a long, narrow test-tube, and carefully sealed
with vaseline. Both the aerated and the sealed bacterial extract were held at
38°C. for 24 hours. Equal portions of the aerated and sealed extract were then
tested for their lipase activity as described in the preceding experiment.

The effect of exposure of the hydrolysis mixture to air during the incubation period allowed for enzyme action was tested as follows: 4.0 cc. of tributyrin solution were placed in each of a series of narrow (11 mm.) test-tubes; the same amount of tributyrin solution was placed in a series of wide (50 mm.) tubes. 1.0 cc. and 0.3 cc. of the sterile bacterial extract (not previously exposed to air) were then added to separate narrow tubes of tributyrin solution; these tubes were then carefully sealed from air by a heavy layer of sterile vaseline. The same amounts of the same enzyme solution were added to the wide tubes of tributyrin; this series was not sealed with vaseline. The two series then represented hydrolysis mixtures, one of which was protected from air by a high column of fluid overlaid with vaseline, while the other was freely exposed to air in a shallow layer. Controls with heat-inactivated enzyme solution were included for both the sealed and aerated series.

TABLE III.

Effect of Exposure to Air upon the Lipase Activity of Sterile Extracts of Botulinus Bacilli.

Amount of bacterial extract	Effect of previous aeration upon subsequent lipase activity (enzyme solution aerated in absence of substrate)		Effect of aeration of the hydrolysis mixture (enzyme aerated in presence of substrate)	
	Change in reaction due to hydrolysis of tributyrin		Change in reaction due to hydrolysis of tributyrin	
	Bacterial extract previously exposed to air for 24 hrs.	Bacterial extract not previously exposed to air	Aerated hydrolysis mixture	Sealed hydrolysis mixture
cc.	ΔpH	ΔpH	ΔpH	ΔpH
1.0	1.5	1.5	1.4	1.4
0.3	0.8	0.8	0.8	0.8

After 60 hours incubation at 38°C., the degree of hydrolysis of the tributyrin was estimated by comparisons of the changes in reaction in the different test mixtures. The results are summarized in Table III.

As shown in Table III, previous aeration of the extract of the bacterial substances was without effect upon the subsequent activity of the lipase, and likewise the presence of air during the incubation of the enzyme-substrate mixture did not influence the action of the enzyme upon tributyrin. The resistance of the lipase to aeration is in striking contrast to the susceptibility of the living anaerobic cells from which the enzyme was derived.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Experiments were carried out to determine whether the maltase and lipase of *botulinus* bacilli are endocellular or exocellular. These experiments consisted in comparisons of the activities of the supernatant fluid of a young unautolyzed broth culture with the activities of a sterile extract prepared by disrupture of the centrifuged bacterial cells of the same culture.

The preceding demonstrations of the lipase and maltase of *botulinus* were made with sterile extracts of the bacilli suspended in the supernatant fluids of broth cultures. Obviously, these enzyme solutions contained both the exocellular sub-

TABLE IV.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Hydrolysis test mixture		
Source of enzymes	Substrate	
	Maltose	Tributyrin
Sterile bacterial preparation containing	Change in pH due to fermentation of glucose formed by *botulinus* maltase	Change in pH due to splitting of tributyrin by *botulinus* lipase
	ΔpH	ΔpH
Only exocellular products of *botulinus* bacilli	0.0	1.0
Both exocellular and endocellular products of *botulinus* bacilli	0.5	1.0

stances liberated during the growth of the culture and the intracellular substances set free by the disintegration of the suspended bacteria. For the following experiment, it was necessary to obtain a sterile solution containing only the exocellular products which are liberated into the culture fluid during the period of active growth and before bacterial dissolution had progressed.

1. Solution Containing Only Exocellular Substances.—The fluid used as the solution of exocellular substances consisted of the supernatant fluid obtained by centrifugation of an 18 hour broth culture.

2. Solution Containing Both Exocellular and Endocellular Substances.—The bacterial solution was prepared from the sedimented bacterial cells of the same culture as that used for Solution 1. The bacilli were suspended in a small amount of the supernatant fluid as described under Methods for the preparation of bacterial extracts, and subjected to the freezing and thawing process.

Both the supernatant fluid and the bacterial solution were finally filtered

through a Berkefeld filter. The comparison of the maltase and lipase activities of the two preparations was made by the procedure used in the preceding experiments. The results are summarized in Table IV.

The results of this experiment (Table IV) show a difference in the nature of the maltase and lipase of *botulinus*. The maltase must be endocellular in nature, since maltose is hydrolyzed only by the sterile preparation which contains the intracellular substances of the bacilli and not by the sterile filtrate which contains only the *botulinus* products of exocellular origin. The lipase, on the other hand, seems to represent an extracellular rather than an intracellular substance, since tributyrin is split as actively by the sterile *botulinus* filtrate which contains only exocellular products as by the extract of the bacterial substances. The agreement in the extent of glyceride hydrolysis by the two *botulinus* preparations indicates that no lipase is contained within the *botulinus* bacilli themselves. Thus, while the endocellular maltase can be accepted as an actual constituent of the *botulinus* bacterial cell, the glyceride-hydrolyzing enzyme must be considered a substance of different nature which the bacillus liberates into the culture fluid during the period of active growth before bacterial autolysis occurs.

DISCUSSION.

This investigation presents experimental evidence of the maltase and lipase of the *botulinus* bacillus and thus demonstrates the enzymatic nature of two biochemical activities of this anaerobic organism. The proof of the maltase not only shows that *botulinus* bacilli possess an enzyme capable of hydrolyzing maltose in the absence of the bacteria themselves, but furnishes evidence that the capacity of these anaerobic bacilli to ferment different disaccharides depends upon their possession of a specific enzyme to hydrolyze the disaccharide to its component hexoses. The sterile bacterial extract prepared from a strain of *botulinus* which ferments maltose, but not lactose and sucrose, contains an active maltase but no lactase or sucrase.

The maltase enzyme, like the carbohydrate-splitting enzymes of certain aerobic bacteria, as pneumococci (5–7) and meningococci (3), is endocellular in nature, and hence is an actual constituent of the *botulinus* bacterial cell. The lipase, on the other hand, is an extracellular substance, and is extruded into the culture fluid during the period of growth of the bacteria. The lipase of *botulinus* differs in this respect from the endocellular one of Pneumococcus (6, 7) but agrees with those from a number of other aerobic bacteria (8) which also are exocellular in nature.

The results of the experiments presented show that the lipase of *botulinus* is resistant to exposure to air. It was found that 24 hours

aeration of the bacterial extract at 38°C. had no detectable effect upon the subsequent activity of this enzyme; similarly, free exposure to air of the enzyme-substrate mixtures during the incubation period allowed for enzyme action did not seem to affect its activity. The indifference to air which is shown by the heat-labile lipase of *botulinus* is in striking contrast to the susceptibility of the living bacillus. If oxygen be considered toxic to anaerobes, the resistance to oxidation shown by the lipase of these anaerobic bacilli is analogous in some respects to other known examples of the relative resistance of individual cell products to deleterious influences which are toxic to the living cell as a whole. Frequent examples are known of enzymes of aerobic bacteria which withstand heating treatment which kills the cell itself; similarly, concentrations of disinfectants which promptly end the life of the bacterial cell as a whole, often have but a slight inhibitory effect upon many of the enzymes derived from the same bacteria. Nevertheless, in spite of the frequency with which somewhat similar phenomena are encountered, it is of considerable interest to observe that the fat-hydrolyzing enzyme of this anaerobic bacillus, after its elaboration, is apparently indifferent to conditions of oxygen tension which render impossible the growth of the bacilli from which the enzyme is derived.

<div align="center">SUMMARY.</div>

Botulinus bacilli yield a maltase and a lipase, which retain their hydrolytic activity independent of the presence of the formed bacterial cell. The maltase is an endocellular substance, while the lipase is extracellular. The lipase, when separated from the anaerobic bacillus, does not seem to be affected by exposure to air.

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STUDIES ON BACTERIAL ENZYMES.

V. The Carbohydrases and Lipase of the Welch Bacillus.

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INTRODUCTION.

Gas production is the most prominent metabolic process of *B. aerogenes capsulatus*—the Welch bacillus—both in test-tube culture and in the animal body. It is desirable, therefore, to obtain further knowledge of the reactions involved in the fermentation of higher carbohydrates by this anaerobic organism. Previous studies on pneumococci (1–3) and meningococci (4) have demonstrated carbohydrate-hydrolyzing enzymes and also their hexose products, and thus constitute proof that hydrolysis represents a preliminary reaction in the fermentation of complex sugars by these particular species of bacteria. However, the products formed in the Welch bacillus fermentations are quite different from those formed from the same sugars by the bacteria with which the hydrolyzing enzymes have actually been demonstrated. This fact, together with the reports in the literature (5) of polysaccharide fermentation by other bacteria without preliminary hydrolysis, makes it desirable to seek experimental proof of the carbohydrases of the Welch bacillus rather than to assume their presence from observations that the specific carbohydrate is fermented by the living bacilli. Conversely, the demonstration of the enzymes themselves and of their hexose products furnishes at the same time indirect evidence that the higher carbohydrate is not attacked without preliminary hydrolysis.

This investigation also deals with the effect of oxidation upon the activity of the Welch bacillus enzymes. The deleterious influence of air upon the growth of strict anaerobic bacteria makes it evident

that oxygen must have a toxic or inhibitory effect upon at least some of the cellular agents involved in life processes of the bacterial cell. While it seems probable that different cellular constituents vary in their susceptibility to air, so many different agents are concerned in the complex functions and processes of "growth" and "life" that it is impossible to determine, with living cultures, the relative sensitivity of the individual components. With "enzyme solutions" or sterile extracts of the bacterial bodies, the various cellular components have already been formed under optimum conditions for growth, and hence can subsequently be tested in respect to their individual susceptibility to oxidation without introducing all of the complications entailed in the "growth" of living cultures of the bacilli.

EXPERIMENTAL.

Methods.

Preparation of the Sterile Bacterial Extracts or Enzyme Solutions.—The sterile extracts or enzyme solutions were prepared from a typical strain of the Welch bacillus by a method essentially the same as that previously described for the preparation of sterile extracts of *botulinus* bacilli (6). The Welch bacilli offer considerable resistance to disintegration by freezing and thawing and it was necessary to repeat the process 150 times to obtain satisfactory extracts. After Berkefeld filtration, the bacterial extract was perfectly clear, but contained a considerable amount of dissolved bacterial substance as indicated by the marked precipitation of coagulated protein when boiled.

Sterility Controls.—No antiseptics were used; the sterility of each enzyme-substrate mixture was controlled by cultural methods.

Substrates.—The solutions of carbohydrates and tributyrin were prepared in the same manner as described in the preceding paper. Unless otherwise specified, air was excluded from all of the hydrolysis test mixtures during the incubation period allowed for enzyme action by preparing them in long, narrow tubes sealed with a heavy layer of vaseline.

Carbohydrate Hydrolysis by Enzymes Contained in Sterile Extracts of Welch Bacilli.

5 cc. portions of sterile, 3 per cent solutions of maltose, lactose, sucrose, raffinose, and starch were distributed into five series of sterile Pyrex test-tubes. 0.2 cc. of the sterile bacterial extract was added to one tube of each of the substrates. A second series was then prepared to serve as controls of the heat lability of the enzymes; the same amount of heat-inactivated bacterial extract was

added to each of these tubes. All of the mixtures were then sealed with vaseline to exclude air, and incubated at 38°C. for 48 hours.

At the end of this incubation period, tests were made for the presence of the products of enzyme action. The sucrose, raffinose, and starch series were tested with Benedict's solution, and the approximate per cent of reducing sugars estimated by comparison with standard glucose solutions. With the lactose and maltose hydrolysis test mixtures (where the substrates themselves are reducing sugars) it was more convenient to detect the hexoses by the biological method described in previous papers (4, 3, 6).

Living cultures of Welch bacilli actively attack the hexoses which would be formed by enzymes hydrolyzing the above substrates. Hence, tests were made

TABLE I.

Splitting of Carbohydrates by Enzymes Contained in Sterile Extracts of Welch Bacilli.

Substrate	Carbohydrate hydrolysis		Evidence of action on hexose products	
	Biological tests for presence of hexose products of enzyme action	Chemical tests for reducing sugars		
	Change in pH due to hexoses previously formed by Welch bacillus enzymes	Per cent reducing sugars	Acid production	Gas production
	ΔpH^1	*per cent*		
Maltose	1.5	Not tested	0	0
Lactose	1.2	Not tested	0	0
Sucrose	Not tested	0.8	0	0
Raffinose	Not tested	0.6	0	0
Starch	Not tested	0.3	0	0

[1] ΔpH when used in Tables I to IV indicates change (decrease) in pH.

to see whether or not the sterile extract of the Welch bacilli produced either acid or gas in the hydrolysis mixtures in which the hexoses had accumulated during the period of enzyme action.

The results are summarized in Table I.

The results of these experiments (Table I) show that sterile extracts of the bacterial substances of Welch bacilli contain enzymes which actively hydrolyze maltose, lactose, sucrose, raffinose, and starch. Living cultures of Welch bacilli attack all of these complex carbohydrates with the vigorous production of acid and gas. In contrast to the living bacteria the sterile extracts are without action upon the

hydrolytic products of the above carbohydrates, as evidenced by their failure to form either acid or gas in mixtures containing a relatively high concentration of the fermentable hexoses. Other tests were made with 1 per cent glucose both in phosphate solution and in broth, and in these tests the bacterial extract was likewise without action upon the hexose which yields both acid and gas in the presence of the living Welch bacillus.

The logical explanation of these phenomena is the same as that previously made for similar results with sterile extracts of pneumococci (3) and meningococci (4). The living Welch bacillus attacks complex carbohydrates through a preliminary splitting to hexoses by a specific hydrolytic enzyme, and then subsequently ferments the hexose by means of other cellular processes. Although these two reactions are probably simultaneous in the living cell, they represent two distinct and separate processes, since only one of them (the hydrolytic property) retains its activity in sterile filtered solutions of the intracellular substances (7). Thus, the above experiment furnishes experimental evidence of the order of reactions operative in the fermentation of complex carbohydrates by Welch bacilli.

The ability of different strains to attack a specific polysaccharide depends upon their possession of the requisite hydrolyzing enzyme. Living cultures of the strain from which the above bacterial extract was derived, vigorously fermented all of the substrates listed in Table II. The same strain is without action upon inulin and similarly the bacterial extract seems to be totally devoid of an inulin-hydrolyzing enzyme.

Splitting of Tributyrin by Sterile Extracts of Welch Bacilli.

While the present paper is concerned principally with carbohydrate-hydrolyzing enzymes, the sterile extracts of Welch bacilli also contain an active enzyme which hydrolyzes tributyrin. Experimental evidence of the splitting of the glyceride by a heat-labile agent derived from the Welch bacillus is presented in the protocol given in Table II.

As shown by these results (Table II), the heat-labile lipase of this anaerobic bacillus retains its ability to hydrolyze tributyrin in the complete absence of living or formed bacterial cells. It is interesting to observe that the Welch bacillus apparently possesses a much less

active lipase than does the *botulinus* bacillus. Comparison with the results given in the preceding paper (6) makes it evident that much larger amounts of the Welch bacillus extracts are required to cause the same degree of hydrolysis than in the case of the enzyme solution

TABLE II.

Splitting of Tributyrin by Sterile Extracts of Welch Bacilli.

Hydrolysis test mixture	Amount of bacterial extract	Reaction of test mixtures after 48 hrs. incubation at 38°C.	Change in reaction due to splitting of tributyrin by enzyme action
	cc.	*pH*	*ΔpH*
Tributyrin plus active bacterial extract	3.0	6.5	0.9
	0.5	7.2	0.4
Tributyrin plus heat-inactivated bacterial extract	3.0	7.4	0
	0.5	7.6	0

TABLE III.

Endocellular Nature of Carbohydrases and Lipase of the Welch Bacillus.

Sterile bacterial preparation	Substrates				Enzyme activity
	Maltose	Sucrose	Starch	Tributyrin	
	Change in pH due to fermentation of glucose formed by Welch enzymes	Reducing sugars by Benedict test	Reducing sugars by Benedict test	Change in pH due to splitting of the glyceride	
	ΔpH	*per cent*	*per cent*	*ΔpH*	
Bacterial extract (filtered solution of intracellular substances)	1.2	0.8	0.3	0.8	+
Filtrate of supernatant of young broth culture (containing only exocellular substances)	0.0	0.0	0.0	0.0	0

derived from *botulinus*. The maltase action of the Welch bacillus preparation, on the other hand, is much more pronounced than was that of the bacterial extract prepared from the *botulinus* bacilli, a relation which is paralleled by the metabolic activities of living cultures of the same bacteria.

Endocellular Nature of the Carbohydrases and Lipase of Welch Bacilli.

The test of the endocellular or exocellular nature of the enzymes consisted in a comparison of the enzyme activity of the bacterial extract containing both intra-cellular and extracellular products with the activity of the culture filtrate which contained only extracellular substances.

The protocol is summarized in Table III.

It is evident (Table III) that the carbohydrases and the lipase of the Welch bacillus are endocellular in nature. Its carbohydrases agree in this respect with the similar enzymes of Pneumococcus (1–3, 8, 10), Meningococcus (4, 9), and the *botulinus* bacillus (6). The Welch bacillus lipase agrees with that of Pneumococcus (8, 10) while it differs from the lipase of *botulinus* which is an extracellular substance not retained within the bacterial cell itself but liberated into the environment during the period of active growth of the bacillus (6).

Our real interest in the endocellular nature of these enzymes of the Welch bacillus is to furnish a basis for following experiments on their oxidation and inactivation by exposure to air. These enzymes have been proved in the above experiment to be endocellular in nature and not liberated into the culture fluid until the bacterial cells are dis-integrated, and consequently, it is necessary to accept them as actual components of the bacterial cell.

Relative Resistance to Oxidation Shown by the Carbohydrases and the Lipase of the Welch Bacillus.

Tests were made of the effect of previous aeration of the bacterial extract upon the subsequent activity of enzymes; tests were also made of the inactivat-ing effect of treatment with different concentrations of hydrogen peroxide, a chemical oxidizing agent which is identical or similar to those involved in many biological oxidations.

One portion of the sterile bacterial extract was freely exposed to air for 30 hours at 38°C. in a shallow layer in an Erlenmeyer flask. Hydrogen peroxide ("dioxogen" diluted in sterile pH 7.5 phosphate solution) was added to a second portion of the same extract in amount sufficient to give a concentration of 10 mM H_2O_2; the same reagent was added to a third portion of the extract to give a concentration of 100 mM H_2O_2; the H_2O_2-treated extract was also stored at 38°C. for 30 hours in long narrow tubes sealed with vaseline. Another portion of the original extract was stored under similar conditions to serve as a control of the

activity of the unoxidized or unaerated extract. A fifth portion of extract was boiled for 10 minutes as a control for all of the hydrolysis mixtures.

For the subsequent tests of maltase and lipase activity, the following hydrolysis mixtures were prepared: (1) substrate plus enzyme solution which had been protected from air; (2) substrate plus the enzyme solution which had previously been exposed to air for 30 hours; (3) substrate plus the enzyme solution exposed to 10 mM H_2O_2; (4) substrate plus the enzyme solution exposed to 100 mM H_2O_2; (5) substrate plus enzyme inactivated by heat (boiled).

The tests of the enzymes attacking sucrose, raffinose, and starch included only three of the above series, and consisted of the following mixtures: (1) substrate plus enzyme solution which had been protected from oxidation; (2) substrate

TABLE IV.

Relative Resistance to Oxidation Shown by the Carbohydrases and Lipase of the Welch Bacillus.

Treatment of bacterial extract	Lipase	Maltase	Sucrase	Raffinase	Amylase
	Change in pH due to splitting of tributyrin by Welch bacillus enzyme	Change in pH due to fermentation of glucose formed by Welch bacillus enzyme	Reducing sugars	Reducing sugars	Reducing sugars
	ΔpH	ΔpH	*per cent*	*per cent*	*per cent*
Bacterial extract stored at 38°C. but protected from air	0.8	1.5	0.9	0.7	0.4
Exposed to air for 30 hrs. at 38°C.	0.9	1.0	Not tested	Not tested	Not tested
Exposed to 10 mM H_2O_2	0.8	0.6	Not tested	Not tested	Not tested
Exposed to 100 mM H_2O_2	0.4	0.0	0.0	0.0	0.0
Boiled for 10 min.	0.0	0.0	0.0	0.0	0.0

plus enzyme exposed to 100 mM H_2O_2; (3) substrate plus heat-inactivated enzyme solution.

All of the above hydrolysis mixtures were prepared in narrow Pyrex tubes and were sealed with vaseline to exclude air during the incubation period allowed for enzyme action. After 72 hours incubation at 38°C., the test mixtures were examined by the methods used in preceding experiments.

The results of the experiment are summarized in Table IV.

As shown in Table IV, the maltase suffered a significant loss in subsequent activity when the bacterial extract was exposed to air for

30 hours, or when it was exposed to a relatively low concentration of H_2O_2 (10 mM, or approximately 0.03 per cent). The lipase, on the other hand, did not seem to be affected at all by either of these two oxidation treatments. In the tests with a higher concentration of H_2O_2 (100 mM), similar relations were evident: while exposure to this oxidation treatment inactivated completely the carbohydrases (maltase, sucrase, raffinase, amylase), the same conditions caused only a partial inactivation of the lipase. These facts are evidence that the lipase of the Welch bacillus is much more resistant to oxidation than are its carbohydrases, whether the oxidative processes are induced by aeration or by treatment with hydrogen peroxide.

Since the carbohydrases and lipase of the Welch bacillus must be accepted as actual constituents of the cell, the above results acquire interest as examples of differences in the relative sensitivity to oxidation which are possessed by the various cellular components of anaerobic bacilli. The greater susceptibility to oxidation is apparently a characteristic of bacterial carbohydrases. The carbohydrate-splitting enzymes of Pneumococcus are much more easily inactivated by oxidation than are their glyceride-splitting and peptone-splitting enzymes (2, 3, 10); similarly, the maltase of meningococci is much more readily oxidized than is the peptonase of the same bacteria (9).

Influence of Aeration of the Enzyme-Substrate Mixture during the Incubation Period Allowed for Enzyme Action.

The preceding experiment dealt with the effect of previous aeration of the bacterial extract itself upon the subsequent activity of the enzyme. The object of the present experiment is to determine whether or not free exposure of the enzyme-substrate mixture to air during the period of enzyme action affects the activity of the same enzymes.

The experiment was similar to that previously made in the investigation of the same question with *botulinus* lipase (6). Two series of both tributyrin-enzyme mixtures and of maltose-enzyme mixtures were prepared. One series was incubated in a narrow tube sealed from air with a heavy layer of vaseline. The second series was exposed to air by placing the enzyme-substrate mixture in a wide tube; this fluid was subjected to free aeration as a wide surface area was exposed and the layer of fluid was shallow (approximately 2 mm.).

Both the aerated series and the series protected from air were incubated for 48

hours at 38°C. The extent of enzyme action was then determined by the methods employed in previous experiments.

The results of the above experiments were conclusive. The lipase, as in the previous study of the similar enzyme of *botulinus*, was indifferent to the presence of air and exhibited the same degree of activity in the aerated hydrolysis mixture as in the mixture protected from air. Even the labile maltase was not affected to a detectable degree by the presence of air in the enzyme-substrate mixture. Although the maltase was inactivated to a significant extent in the previous experiment where the undiluted bacterial extract was aerated before its addition to the substrate, the active enzyme itself hydrolyzed maltose to the same extent when the enzyme-substrate mixture was exposed to air as when air was excluded during the period of enzyme action.

While the maltase appears to be relatively indifferent to aeration of the enzyme-substrate mixtures during the period of enzyme action, it is partially inactivated when the bacterial extract itself is exposed to air previous to the preparation of enzyme-substrate mixtures. Sufficient data are not at hand to explain this apparent difference in the resistance of the maltase to the two conditions of aeration. Other workers (11) have reported similar differences in the destruction of enzymes, where the enzyme inactivation has been less pronounced if the substrate is present during the exposure of the enzyme to a deleterious influence than if the enzyme is subjected to the same conditions in the absence of the substrate. While the above phenomenon in the case of Welch bacillus maltase may also be due at least in part to a similar "protective action of the substrate," we believe that an important factor in our experiment is the difference in the dilution of the bacterial extract under the two sets of conditions. In the first instance, air was allowed to act upon the undiluted bacterial extract; in the second experiment, where the enzyme-substrate mixture was exposed to air, the bacterial substances were present in only about one-tenth their original concentration. If the inactivation of the maltase through oxidation by air is not a first order reaction, the dilution itself would decrease the speed of the inactivating processes.

DISCUSSION.

The hydrolysis of tributyrin and of a number of different carbohydrates by heat-labile agents present in sterile filtered solutions of the intracellular substances of the Welch bacillus proves that the action of its lipase (tributyrinase) and carbohydrases (maltase, lactase,

sucrase, raffinase, amylase) is independent of the presence of either the living or formed bacilli. The further study of these enzymes can contribute to our knowledge of the mechanism of metabolism and method of growth of this pathogenic organism.

The results of the experiments on the carbohydrases show that the fermentation of complex carbohydrates, a characteristic metabolic process of the living Welch bacillus, is dependent upon the presence of specific hydrolyzing enzymes. Since the sterile bacterial extract which contains the active hydrolases is devoid of any action upon the hexose products, it is possible to detect intermediate products which would be rapidly consumed in experiments with the living bacilli. Thus, the above demonstration of the carbohydrate-hydrolyzing enzymes of the Welch bacillus constitutes experimental evidence that Welch bacilli attack complex carbohydrates by way of two separate sets of reactions: (1) hydrolysis; (2) fermentation of the hexose products of the hydrolytic reaction. Of the two reactions (hydrolysis and fermentation) which are effected by the living cell, only the hydrolytic property retains its activity in sterile, filtered solutions of the bacterial substances.

Exposure of the bacterial extract to air resulted in no detectable loss in the subsequent activity of the lipase, but caused a slight loss in the activity of the more labile carbohydrases. Treatment of the bacterial extract with a low concentration of hydrogen peroxide, although entirely without effect upon the lipase, caused a partial inactivation of the maltase; a higher concentration of peroxide which was sufficient to induce only a partial inactivation of the lipase caused the complete inactivation of all of the carbohydrases. Thus, although both the lipase and the various carbohydrases are affected by sufficiently strong oxidation treatment, the lipase is more resistant than the carbohydrases whether the oxidative processes be induced by air or by hydrogen peroxide. The lipase and carbohydrases are intracellular substances and hence must be considered as integral constituents of the Welch bacillus cell. From this point of view, the greater sensitivity of the Welch bacillus carbohydrases in comparison to the lipase presents the first real demonstration of the differences in susceptibility to oxidative inactivation which might be expected to exist between individual cellular components of anaerobic bacteria.

Although exposure of the anaerobic extract to air previous to its addition to the substrate caused a slight but definite inactivation of the carbohydrases, air had no effect when the exposure was made in the presence of the substrate during the period of enzyme action.   In fact, so far as we could determine, all of the enzymes (the more labile carbohydrases as well as the lipase) hydrolyzed their substrates as effectively in the presence of air as in its absence.   The indifference of the enzymes to air under these conditions is in striking contrast to the extreme susceptibility of the living cell from which the enzymes were derived.   The fact that the presence of air inhibits growth of strict anaerobic bacteria makes it evident that oxygen must have a toxic or inhibitory effect upon at least some of the cellular agents involved in certain life processes of the anaerobic cell.   Since the various components of a cell differ in their individual resistance toward deleterious agents, the inhibition of growth of the cell as a whole would be determined by the specific sensitivity of the most labile component, if this be involved in a process inherently essential to the life of the organism.   One would be inclined to limit the category of indispensable life processes to the energy-productive reactions concerned in the initiation of growth of the cells.   Since the hydrolyzing enzymes are not directly concerned in reactions of this type, it is extremely unlikely that their functions would ultimately determine the growth of the culture in any environment.   However, in spite of these relations, it is of interest to obtain experimental evidence that the inhibitory effect of air upon the initiation of growth of the Welch bacillus does not include a comparable effect upon the activity of its hydrolyzing enzymes.   In fact, the evidence indicates that there is no essential difference between the hydrolytic enzymes of aerobic and anaerobic bacteria in respect to sensitivity to oxidation.

The slight degree of inactivation of the carbohydrases when an extract of Welch bacilli is exposed to air is by no means comparable to the rapid and almost complete inactivation of carbohydrases which occurs under the same conditions in a similarly prepared extract of pneumococci.   The apparent difference in the effect of air upon the carbohydrases contained in extracts of pneumococci and of Welch bacilli is not in itself important, but it is desirable to analyze the factors involved since the same fundamental principles are concerned in

the oxidation of many other biological substances. When oxidative inactivations take place in complex biological systems, an explanation of apparent differences in the resistance of substances in these systems (12) requires the consideration of at least two factors: (1) the intrinsic susceptibility to oxidation of the substances themselves; (2) the oxidizing activity of the systems in which the substances are exposed to air.

In the present instance, there is no evidence to support the possibility of differences in the intrinsic susceptibility to oxidation of the carbohydrases derived from the two bacteria, but there is convincing evidence that the two *systems* in which the carbohydrases are contained differ materially in oxidizing activity. First, peroxides are formed in significant and easily detectable concentrations in aerated pneumococcus extracts (13), while similar oxidizing agents, if produced in the Welch bacillus extract, do not accumulate in detectable amounts. Second, when a known substance, hemoglobin, is added to the two systems, it is likewise oxidized much more rapidly and more completely in the system (pneumococcus extract (7, 14)) in which the oxidation of the enzyme is more pronounced. Finally, when the system in which the pneumococcus enzymes are contained is changed to one in which peroxides are not formed (by preparing an extract from washed cells suspended in salt or phosphate solution (2)) the pneumococcus carbohydrases are fully as resistant to air as are those of the Welch bacillus. The above analysis is of interest as a definite illustration of the importance of the oxidizing activity of the *system* in determining the resistances of different biological substances to air; the same factor is probably involved in other instances where the oxidizing activity of the complex system is not so easily recognized.

SUMMARY.

The carbohydrases (maltase, lactase, sucrase, raffinase, amylase) and lipase (tributyrinase) of the Welch bacillus retain their hydrolytic activities in sterile solutions of the bacterial cells. The demonstration of the carbohydrases and detection of their hexose products constitute experimental proof that Welch bacilli attack complex carbohydrates by way of a preliminary hydrolysis.

The results of experiments on the oxidation of the enzymes show that the inhibitory effect of air upon the initiation of growth of the living Welch bacillus does not include a comparable effect upon the activity of its hydrolyzing enzymes.

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VARICELLA IN MONKEYS.

NUCLEAR INCLUSIONS PRODUCED BY VARICELLA VIRUS IN THE TESTICLES OF MONKEYS.

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The lesions observed in testicles of monkeys inoculated with emulsified human varicella papules and vesicles have been described in a previous paper (1). In the experiments reported at that time several species of monkeys were employed and also rats, rabbits, guinea pigs, and chickens. Significant lesions, nuclear inclusions, were found only in the testicles of African vervets 5 and 6 days after inoculation, and not in other inoculated tissues of the same animals, nor in the other experimental animals. Furthermore, similar inclusions were not found in the testicles of a vervet inoculated with normal skin. The inclusions were the eosin-staining nuclear bodies which are consistently associated with certain virus diseases and which, regardless of their nature, indicate to many workers the presence of a virus. Therefore, in view of these facts, it was deemed not unlikely that the acidophilic nuclear inclusions in the vervets' testicles were manifestations of the presence of a virus. The nature of the virus had not been studied at the time of the previous report. Recently, however, experiments were performed to obtain, if possible, additional information concerning the suspected virus and it is with the results of this work that the present paper deals.

Methods and Materials.

Monkeys Employed.—It was impossible to obtain vervets (*Cercopithecus lalandi*). A search for susceptible animals was made among other *Cercopithecus* monkeys. A few experiments showed that green monkeys (*Cercopithecus sabæus*) very closely allied to vervets were satisfactory for the work. It is essential that the monkeys be young, and all animals in which spermatogenesis had been established were discarded.

Inoculations.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5–1.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of Locke's solution, non-immune serum, or immune serum. The mixtures were then injected into monkeys. More than 45–60 minutes never elapsed between the collection of the virus from the patients and its inoculation into animals. All monkeys were inoculated intratesticularly (0.2–2.5 cc.). The non-immune serum was obtained from varicella patients usually within the first 72 hours of the disease. The immune serum was obtained from convalescent varicella patients 14–22 days after the onset of the disease.

Removal and Examination of Testicles.—In previous experiments (1) it was determined that nuclear inclusions were present in the testicles on the 5th and 6th days after inoculation. Therefore in the experiments reported at the present time the monkeys were castrated* on the 5th day. Testicles removed for histological studies were fixed in Zenker's fluid with 5 per cent acetic acid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (2), Lipschütz (3), Goodpasture (4), and others.

EXPERIMENTAL.

The experiments to be reported were conducted to determine whether the eosin-staining nuclear inclusions in monkeys' testicles inoculated with emulsified varicella papules and vesicles are specifically associated with the virus of varicella. In connection with this phase of the work six experiments, consisting of neutralization and reinoculation tests, were performed, and a detailed account of each is given below.

Experiment 1.—Monkeys L and M; green. December 23, 1925. 2 lesions were removed from each of 3 varicella patients, Cases 18, 19, and 20, 3 days after the onset of the disease. The papules and vesicles were emulsified together and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Case 18 was bled on the 3rd day of the disease for non-immune serum; Case 17 was bled for immune serum 17 days after onset of the disease. Equal amounts of the sera, not inactivated, were mixed respectively with the two portions of emulsified papules and vesicles. The mixtures were injected immediately

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* All operative procedures were conducted under ether anesthesia.

into 2 green monkeys as follows: Monkey L, 0.25 cc. of virus and immune serum in each testicle; Monkey M, 0.25 cc. of virus and non-immune serum in each testicle. The 4 testicles, removed 5 days later, were fixed and examined in the usual way for the presence of eosin-staining nuclear inclusions.

Although nuclear inclusions were not found in the testicles of Monkey L inoculated with virus and immune serum, typical ones were observed in the testicles of Monkey M inoculated with virus and non-immune serum.

Experiment 2.—Monkeys N and O; green. January 4, 1926. 5 lesions, vesicles and papules, removed from a varicella patient, Case 22, 48 hours after the appearance of the rash were emulsified and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Non-immune serum was also obtained from Case 22 and was inactivated at 56°C. for 30 minutes. Convalescent serum from Case 17, collected and inactivated, December 23, 1925, was used as the immune serum. Equal amounts of the sera were mixed respectively with the two portions of virus emulsion. 2 green monkeys were inoculated immediately; Monkey N, 0.25 cc. of virus and immune serum in each testicle; Monkey O, 0.25 cc. of virus and non-immune serum in each testicle. Monkey N also received 3 cc. of the convalescent serum intraperitoneally; Monkey O, 3 cc. of the non-immune serum in a similar manner. 5 days later the monkeys were castrated. The testicles were fixed, sectioned, and examined in the usual way for the presence of nuclear inclusions.

Nuclear inclusions were not observed in the testicles of Monkeys N and O. No explanation of their absence from the testicles of Monkey O has been found.

Experiment 3.—Monkeys P and Q; green. January 8, 1926. 5 lesions, vesicles and papules, were removed from varicella patients,—2 from Case 24 on the 4th day of the disease, 3 from Case 25 within 36 hours after the appearance of the rash,—and ground up together. The emulsified material was taken up in 0.5 cc. of Locke's solution and divided into equal portions. The inactivated non-immune serum was a mixture of sera collected from Cases 18, 22, 24, and 25 on the 3rd, 2nd, 4th, and 2nd days of the disease respectively. The inactivated immune serum was a mixture of sera collected from Cases 17, 18, 21, and 23 on the 17th, 16th, 14th, and 14th days respectively after the appearance of the rash. Equal amounts of the non-immune and immune sera were mixed respectively with the two portions of virus emulsion. The mixtures were injected into 2 green monkeys as follows: Monkey P, 0.25 cc. of virus and immune serum in each testicle; Monkey Q, 0.25 cc. of virus and non-immune serum in each testicle. Monkey P also received 7 cc. of the immune serum intraperitoneally; Monkey Q,

7 cc. of the non-immune serum in a similar manner. 5 days later the testicles were removed, fixed, and examined in the usual way for the presence of nuclear inclusions.

Eosin-staining nuclear inclusions were found in the sections from Monkey Q inoculated with virus and non-immune serum. None were seen, however, in the sections from Monkey P inoculated with virus and immune serum.

Experiment 4.—Monkey A (1); vervet. November 12, 1924. 10 cc. of blood collected from a varicella patient, Case 1, 36 hours after the appearance of the eruption, was injected intravenously into Monkey A. Fluid from 30 vesicles was also collected at the same time and injected intradermally in left eyelid, in left and right thighs, and in right side of abdominal wall. While under observation the animal showed no manifestations suggestive of chicken-pox. No tissue was removed at this time for histological study.

Monkey A (1) was inoculated again, April 7, 1925. Emulsified varicella vesicles and papules from Cases 13 and 14 were injected into the right testicle. 6 days later the testicle was removed and fixed. Nuclear inclusions were not found in the sections.

Monkey A; Monkey R, green. January 9, 1926. 3 lesions were removed from varicella patients,—2 from Case 26 on the 2nd day of the disease, 1 from Case 27 on the 4th day of the disease,—emulsified, taken up in 0.5 cc. of Locke's solution. 0.25 cc. of the emulsion was injected into the left testicle of each monkey. Monkey R also received 7 cc. of fresh unclotted blood from Case 26. 5 days later the inoculated testicles were removed and fixed. A search was made in the usual manner for the presence of nuclear inclusions.

Monkey A was considered to be immune because of the two previous inoculations. Monkey R was normal and approximately as old as Monkey A. Nuclear inclusions were found in the normal testicle inoculated with varicella virus. None were seen, however, in the immune testicle inoculated with the same virus.

Experiment 5.—Monkey R, green. January 9, 1926. Monkey R was employed in Experiment 4. At that time the left testicle was inoculated with varicella virus. It was removed 5 days after the inoculation and sections of it showed nuclear inclusions.

Monkeys R, S, and T; green. February 3, 1926. 2 papules and 2 vesicles were removed from a varicella patient, Case 28, within 48 hours after the appearance of the rash, emulsified, and taken up in Locke's solution. There was 0.75 cc. of emulsion. The non-immune serum was collected from Case 25 on the 2nd day of the disease and inactivated. The immune serum was collected from

the same patient 12 days later and inactivated. 0.25 cc. of the virus emulsion was inoculated in the right testicle of Monkey R. 0.25 cc. of the emulsion was mixed with 0.25 cc. of the non-immune serum and then half of the mixture was injected into each testicle of Monkey S. 0.25 cc. of the virus emulsion was mixed with an equal amount of immune serum and then half of the mixture was injected into each testicle of Monkey T. In addition to the above injections Monkeys S and T received intraperitoneally 3.5 cc. of the non-immune and immune sera respectively. The testicles were removed and fixed 5 days later. A careful search through numerous sections revealed the presence of nuclear inclusions in both testicles of Monkey S. None were seen, however, in the testicles of Monkeys R and T.

Experiment 5, in which reinoculation and neutralization tests were conducted simultaneously, is especially interesting. Monkey R's left testicle inoculated with varicella virus, January 9, 1926, showed nuclear inclusions 5 days later.. In the right testicle, however, inoculated 25 days later with varicella virus no inclusions were found. The non-immune and immune sera used in the neutralization tests were obtained from the same patient; the former on the 2nd day of the disease, the latter on the 14th. The non-immune serum mixed with virus did not prevent the formation of nuclear inclusions in the testicles of Monkey S. On the other hand, the immune serum inhibited the production of inclusion bodies in the testicles of Monkey T.

Experiment 6.—Monkeys U and V; green. April 6, 1926. 5 lesions, vesicles and papules, were removed from a varicella patient, Case 30, within 48 hours after the appearance of the eruption, and emulsified. The volume of the emulsion was made up to 0.5 cc. with Locke's solution. Patient 30 also supplied the non-immune serum. The immune serum was obtained from Case 29 on the 22nd day after the onset of the disease. Both sera were fresh and not inactivated. 0.25 cc. of each serum was mixed with equal amounts of the virus emulsion. The mixtures were injected immediately into 2 green monkeys as follows: Monkey U, 0.25 cc. of virus and immune serum in each testicle; Monkey V, 0.25 cc. of virus and non-immune serum in each testicle. In addition to the above injections, the monkeys received intraperitoneally 3 cc. of the immune and non-immune sera respectively. The monkeys were castrated 5 days later and a search for the presence of nuclear inclusions in the testicles was made in the usual way.

Eosin-staining nuclear inclusions were found in both of Monkey V's testicles inoculated with a mixture of virus and non-immune serum. On the other hand, inclusion bodies were not seen in Monkey U's testicles inoculated with a mixture of virus and immune serum.

The results of the six experiments described above are summarized in Table I.

TABLE I.

Summary of the Results of Reinoculation and Neutralization Tests.

Experi-ment	Monkey	State of monkey	Inoculum	Nuclear inclusions.
1	L	Normal	Virus + convalescent serum	—
	M	Normal	Virus + non-immune serum	+
2	N	Normal	Virus + convalescent serum	—
	O	Normal	Virus + non-immune serum	—
3	P	Normal	Virus + convalescent serum	—
	Q	Normal	Virus + non-immune serum	+
4	A	Actively im-munized	Virus	—
	R	Normal	Virus	+
5	R	Actively im-munized	Virus	—
	T	Normal	Virus + convalescent serum	—
	S	Normal	Virus + non-immune serum	+
6	U	Normal	Virus + convalescent serum	—
	V	Normal	Virus + non-immune serum	+

+ indicates presence of nuclear inclusions in testicles.
— " absence " " " " "

DISCUSSION.

Neutralization and reinoculation tests are the accepted means employed to identify viruses and have been extensively used in investigations concerning vaccine virus (5), variola virus (5), poliomyelitis virus (6), Virus III (7–9), herpes virus (10, 11), and others. Technical details of the tests may vary somewhat with each virus, yet in every instance the usefulness of the tests is dependent either upon the specific virucidal properties of an immune serum or upon a specific refractory state of an immune animal.

Many viruses produce characteristic macroscopic lesions in animals, or cause marked changes in their condition. Such alterations and lesions serve as indications of virus activity. Under proper con-

ditions the occurrence of these changes is specifically prevented either by mixing the virus with a homologous immune serum prior to the inoculation of the animal or by injecting the virus alone into an animal previously immunized by means of the same virus. In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as indicated by the presence of inclusion bodies in the nuclei and cytoplasm of injured cells. Sometimes, however, the microscopic changes are the only manifestation of the presence of a virus (1, 12). When such a condition arises, there is no obvious reason why the microscopic changes, inclusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed.

Many workers believe that the eosin-staining nuclear inclusions are the manifestations of the presence of certain filterable viruses, including the virus of varicella. Consequently at the time of the previous report (1) there were grounds for the belief that the nuclear inclusions found in the monkeys' testicles inoculated with emulsified varicella papules and vesicles were produced by the action of varicella virus. Proof of this, however, was obtained only recently by means of the experiments reported above, and consists, in brief, of the following considerations. Nuclear inclusions were not found in monkeys' testicles inoculated with a mixture of varicella virus and convalescent varicella serum. On the other hand, they were found in testicles inoculated with a mixture of virus and non-immune serum collected from varicella patients early in the disease. Furthermore, the inoculation of one testicle with varicella virus prevented the formation of nuclear inclusions in the other one when it was inoculated at a later date with the same virus.

So far as is known, the evidence presented in this paper is the only definite proof on record that experimental animals are susceptible in any way to the action of varicella virus.

CONCLUSION.

The eosin-staining nuclear inclusions found in the monkeys' testicles inoculated with emulsified tissue of human chicken-pox lesions are specifically associated with the virus of varicella.

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PHYSICAL, CHEMICAL, AND BIOLOGICAL STUDIES ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

COMPARISON WITH THE VIRUS OF FOOT-AND-MOUTH DISEASE.

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In an earlier paper[1] attention was drawn to the resemblances between the effects produced by the incitant of foot-and-mouth disease and of vesicular stomatitis. The characteristics to be especially mentioned are: propagation in guinea pigs in continuous series,[2] the correspondence of the experimental disease produced, and the possibility of transmitting vesicular stomatitis to cattle and swine. Such slight discrepancies as the infrequency of secondary lesions were ascribed to variability of strains of the virus. The two viruses were found to show parallel responses to selective filterability. On the other hand, certain clear-cut distinctions were noted, such as want of cross-immunity in guinea pigs, cattle, and swine, and the failure of the horse to respond to inoculation with the virus of foot-and-mouth disease, while being highly susceptible to the virus of vesicular stomatitis.

The present article deals with experiments on the physical, chemical, and biological characters of the virus of vesicular stomatitis, which may serve as a basis for wider comparison with that of foot-and-mouth disease, and at the same time provide data bearing on taxonomy. The latter subject has received, until the present, scant attention.

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[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx (N.S. xxiii), 147. The Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry should also be consulted.

[2] This has also been done with vesicular stomatitis virus by Cotton, W. E., *J. Am. Vet. Med. Assn.*, 1926, lxix (N.S. xxii), 313.

Description of the Strain of Virus Employed.

Source of Material.—Through the kindness of Dr. John R. Mohler, Chief of the United States Bureau of Animal Industry, and of Dr. W. E. Cotton, Assistant Superintendent of the Bureau's Experiment Station at Bethesda, Maryland, a sample of virus designated as that of vesicular stomatitis was received early in October, 1926. The specimen consisted of lingual vesicle coverings of a cow at the experiment station, which was inoculated in turn with material collected from a New Jersey cow ill with the disease. An extensive epidemic of vesicular stomatitis was raging at the time in northern New Jersey, and in the vicinity of Port Jervis, New York. The virus was active, for Dr. Cotton reproduced the disease in test horses in 20 hours. The material was sent in 50 per cent glycerol. A fragment 2 $\times$ 4 mm. was removed from the glycerol, washed in phosphate buffer at pH = 7.5,[3] (ground with sterile sand, and suspended in the buffer solution. About 0.1 cc. of this was injected by the method described[4] intradermally in the posterior pads of two guinea pigs. After 48 hours both animals showed the typical primary, or inoculation vesicles of experimental vesicular stomatitis, already described.[1] Up to the present the virus has been propagated in guinea pigs through at least 90 consecutive passages.

The clinical course of the affection in these animals was a counterpart of experimental foot-and-mouth disease. Secondary vesicles appeared in uninoculated pads in about half the animals; none was observed in the mouth or on the tongue. In these respects, we confirmed our earlier observations.[1] But the irregular occurrence of secondary lesions produced by this strain of virus does not indicate an essential difference between it and the virus of foot-and-mouth disease. We have already stated[1] that certain samples of foot-and-mouth disease virus may act in this manner in guinea pigs or in cattle.

In general, it may be stated that, apart from the likeness of the clinical course, other effects in guinea pigs of this strain of vesicular stomatitis virus are identical with those of the virus of foot-and-

[3] Phosphate buffer, as mentioned here and elsewhere in this article, is made by adding 2.5 gm. of potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

[4] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, and the Bureau Report.[1]

mouth disease.[5] For example, the mortality rate among 450 guinea pigs showing vesicular stomatitis was about 1 per cent; the affection is also practically non-lethal. The virus was free from constant, visible microorganisms, and on two occasions, when secondary, chance, microscopic bacteria were found admixed with the virus, they could be removed by Berkefeld filtration without injury to the specifically active agent. On the other hand, after inducing its specific effect, the latter invited invasion of ordinary bacteria—a character common to filter-passing viruses generally—so that a vesicle turned pustule on the 3rd to 4th day. Furthermore, in about 95 per cent of the animals the period of incubation was 18 to 48 hours, and the period became shorter and the severity of the disease increased as the concentration of the virus was augmented. Moreover, blood withdrawn 20 to 24 hours after intradermal inoculation was active. Aspirated vesicular contents showed the presence of virus in greatest concentration when this material was obtained from lesions up to 24 hours old. From this time to 72 hours a gradual diminution in virulence took place until after 3 days, when the vesicular contents, or ground infected pad tissues, were only exceptionally active. Finally, resistance of recovered animals to reinoculation with active material from the same source was marked, and the immunity lasted for at least $4\frac{1}{2}$ months.

Hence this sample of the virus of vesicular stomatitis is similar to the one already described[1] and induces in guinea pigs effects indistinguishable from those of the virus of foot-and-mouth disease.

Propagation in Rabbits.

The transfer of vesicular stomatitis to rabbits either has not been attempted, or has been unsuccessful and therefore not reported.

[5] The articles mentioned in Foot-note 12 should be consulted for a description of the effects of the virus of foot-and-mouth disease in guinea pigs. Since it is forbidden to work with the latter in the United States, no direct comparison could be made employing the same stock of normal guinea pigs, and the same laboratory conditions in the case of both viruses. For this reason no cross-immunity tests could be made here, although the results of a wide experience with another strain of the virus of vesicular stomatitis have already been reported.[1]

The rabbit appeared important for making additional tests on the above two viruses, and, incidentally, on another virus, namely, that of febrile herpes.

Comparison with Foot-and-Mouth Disease.—Gins and Fortner[6] found that rabbits could be infected with guinea pig virus by scarification of the mucous membrane of the inner lip surface. Vesicles appeared, and the contained fluid was in turn active for normal rabbits or for guinea pigs. Sixteen consecutive rabbit passages were thus effected. Nicolau and Galloway,[7] employing guinea pig virus, were able to induce local vesicles after intralingual injections. In neither case was there evidence of secondary lesions nor of generalization of the vesicular process.

We studied the susceptibility of rabbits to vesicular stomatitis by injecting the virus in the brain, cornea, and buccal mucous membranes.

The rabbits failed to show any untoward effect after intracranial injection of the active Berkefeld V filtrate obtained from guinea pig pads, which was in the ninth guinea pig passage. In another test, virus was employed which was adapted to rabbits by successful corneal inoculation. The materia of the third, fourth, and fifth corneal passages was kept in 50 per cent glycerol. The glycerol was renewed weekly three times, the corneal tissue was then removed, washed, suspended in saline solution, and injected intracranially. While the injected material was active for the pads of guinea pigs, it failed to affect rabbits upon intracranial inoculation.

It appears, therefore, that the virus of vesicular stomatitis, like that of foot-and-mouth disease,[8] is non-neurotropic. Furthermore, in sharp contrast to the effects of herpetic virus, none of the guinea pigs injected with the virus of vesicular stomatitis showed microscopic evidences of damage to the nervous tissues.

On the other hand, the rabbit reacts specifically to corneal inoculations.[9] Beginning with filtered suspensions of infected pads of the

[6] Gins, H. A., and Fortner, J., *Berl. tierärztl. Woch.*, 1926, xlii, 89; *Centr. Bakt., 1. Abt., Ref.*, 1925, lxxviii, 576.

[7] Nicolau, S., and Galloway, I.-A., *Compt. rend. Soc. biol.*, 1925, xciii, 1283.

[8] For the non-neurotropic effects of foot-and-mouth disease virus consult Levaditi, C., Alberta-Lorente, R., and Galloway, I., *Compt. rend. Soc. biol.*, 1926, xcv, 387.

[9] For mode of inoculation see Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

guinea pig, and later employing the ground corneal scrapings, all of thirteen rabbits, during five consecutive passages, showed typical vesicles along the lines of incision of the cocainized cornea. The lesions appeared from 2 to 4 days after application of the virus and were accompanied by conjunctival inflammation. The vesicles coalesced as a rule, and in two rabbits left small, localized opacities. There was no fever at any time. After 7 days healing began, so that within 2 weeks the eye returned to normal. 10 days later the recovered corneæ were refractory to reinoculation. As little or no work was done in this way with foot-and-mouth disease virus, no comparisons can be made.

As is the case with foot-and-mouth disease virus, scarification of the inner surface of the lips and cheek of the rabbit, and application of filtered suspensions of infected pads of guinea pigs led, within 24 to 48 hours, to distinct, localized vesicles. The histopathology of the lesion is similar to that of the guinea pig pads from experimental vesicular stomatitis and foot-and-mouth disease.

The rabbit, therefore, is susceptible to the virus of vesicular stomatitis, and where comparisons can be made with that of foot-and-mouth disease in the same animal, its effects appear to be identical. The rabbit and the guinea pig are epitheliotropic, but not neurotropic toward the viruses.

Comparison with Herpes.—A limited number of experiments was made with the H. F. strain of herpetic virus described by Flexner and Amoss.[9] This virus, injected intracranially in a rabbit, produced the typical cerebral symptoms and death in 4 days. A portion of the base of the brain was suspended in saline solution equal to a 10 per cent suspension, and of this 0.1 cc. was injected intradermally in the posterior pads of three guinea pigs. In general, the effects were similar to those described by Gildemeister and Herzberg.[10] Edema of the pads was noted, and this persisted for about 4 days, when vesicles appeared. These were often pustular from the start, coalesced, tended to necroses, and lasted for about a week. In addition the frequent occurrence of gangrene in the phalangeal and metatarsal areas, and in all of ten guinea pigs injected through three consecutive passages, retention of urine and feces, and paralyses of the posterior extremities were present. The nervous symptoms first appeared about 5 to 6 days after injection. Only one of the ten animals survived. No secondary vesicles were observed.

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[10] Gildemeister, E., and Herzberg, K., *Deutsch. med. Woch.*, 1925, li, 97.

These comparisons show the herpetic virus to be distinct from the viruses of vesicular stomatitis and of foot-and-mouth disease. These differences are emphasized by the fact that guinea pigs recovered from experimental vesicular stomatitis are susceptible to the herpetic virus and *vice versa*.

The reactions of rabbits to corneal inoculation of the herpetic and of the vesicular stomatitis virus differ. In the former the affection induced is much more severe locally, and encephalitic signs may become evident; in the latter, the lesions in the eye are ordinarily mild, tend to complete and rapid recovery, and no nervous manifestations are observed. Cross-immunity is absent in the recovered animals.

Histopathology of Experimental Vesicular Stomatitis of the Guinea Pig.

In pads of guinea pigs inoculated intradermally with filtered or unfiltered material containing the virus of vesicular stomatitis, the first changes noted are swelling and thickening of the epidermis and derma, as a result of edema and cellular infiltration. The infiltrating cells consist mainly of polymorphonuclear neutrophils (the so called pseudo-eosinophils) and less numerous, monocytes (or macrophages or endothelial leucocytes). After 18 to 24 hours vesicles appear between the epithelial layer and corium, and between the horny and Malpighian strata. These layers are filled with serum at first, but soon cells, especially the neutrophils, invade the fluid. In the corium, however, the vesicles may be filled with blood, with an eventual greater cellular infiltration. At this stage occur active mitosis and striking intranuclear changes, to be described immediately. After 48 to 72 hours, necrosis of epithelial cells is observed and retrogression begins; the vesicles are filled with multinucleated cells and later with granular material and droplets of various sizes. They now begin to contract and to dry. After 3 to 4 days, the horny layer exfoliates, a large number of neutrophils is present, and with this a proliferation of epithelial cells about the site of the vesicle. Thereafter healing begins and the lesion is covered with a scab composed of leucocytes and epithelial cells. After the 8th day, as a rule, the cells of the epidermis appear normal for the most part, and the vesicles are replaced by a highly vascularized granulation tissue.

The nuclear changes of the epithelial cells in the Malpighian layer,

in infiltrating cells of the corium and of the vesicles consist in a condensation of the chromatin about the wall, thus leaving only a shadow of nuclear structure which is surrounded by a darker staining, denser membrane. The nucleoli and nucleolar degeneration particles are stained bluish by Giemsa's or the eosin-methylene blue methods. Within the membrane may be seen, in specimens stained by Giemsa's method, one or more perfectly rounded, light pinkish staining bodies, about 1.5 microns in diameter. Some are smaller, but rarely are there any larger. These changes are most marked in lesions 24 hours old, and are comparable to the so called inclusion bodies described by Gins[11] as characteristic of foot-and-mouth disease. In lesions which are 48 hours old, a large number of somewhat different bodies are noted. They are round or oval and vary in diameter from 2 microns to a size large enough to fill almost the entire nucleus with the exception of a narrow, clear zone between the body and nuclear membrane. With Giemsa's or eosin-methylene blue stains, they appear pink to red in contradistinction to the blue nuclear membrane, and, as a rule, lie on a clear background. Some nuclei may contain from two to four of the smaller sized bodies. In general, they are similar to but not necessarily identical with the intranuclear inclusions which characterize certain other filter-passing viruses.

To summarize, the histopathology of experimental vesicular stomatitis is identical with that of experimental foot-and-mouth disease, described by several observers, notably by Gins,[11] and by Levaditi and his coworkers.[8] Furthermore, we have found indistinguishable pathological conditions in foot-and-mouth disease, not only of guinea pigs, but also of cattle and swine. Finally, it is important to note that the virus of vesicular stomatitis can be classified as one of a group of ultramicroscopic agents, the effects of which are characterized by the presence of peculiar intranuclear changes. Further studies by Dr. Rivers on their significance are in progress.

Titration of Virus.[12]

The virus of vesicular stomatitis, as it exists in ground infected guinea pig pads, or in aspirated vesicular contents, could be diluted 1:10,000.000, but not higher,

[11] Gins, H. A., Centr. Bakt., 1. Abt., Orig., 1922, lxxxviii, 265.

[12] In this, as in other experiments, the technical procedures of experiments are omitted. For details of methods, see Olitsky, P. K., and Boëz, L., J. Exp. Med., 1927, xlv, 673, 685, 815, 833, and the Bureau Report.[1]

and still show activity. In filtered material a 1:10,000,000 dilution also induced the experimental disease in guinea pigs. In the blood of this animal, withdrawn 20 to 24 hours after pad inoculation, the virus was present in much lower concentration: the limit of infectiousness being 1:2000 to 1: 200,000.

According to these tests which show the limit of infectiousness at 1:10,000,000 in filtered or unfiltered material, the virus of vesicular stomatitis corresponds in activity to that of foot-and-mouth disease.[4]

Failure of Sedimentation of the Virus.

Centrifugation, at 3700 revolutions a minute for 2 hours, of material containing the virus of vesicular stomatitis failed to bring about its sedimentation. In graded dilutions of 1:30 to 1:300,000, the topmost and the lowest layers behaved in all instances alike. In this respect also the two viruses are similar.[4]

Selective Filtration through Chamberland Bougies.

In an earlier paper[1] it was stated that the behavior of the virus of vesicular stomatitis on filtration paralleled that of the virus of foot-and-mouth disease. Both passed readily through Seitz' asbestos discs and Berkefeld V candles, but not always through Berkefeld N filters. The same was true in the case of Chamberland bougies, both viruses passing through the L 3 and L 7, but usually not through the L 11 type.[13] Hence it was concluded that the two viruses had the same tendency to adsorption in the walls of denser, electronegative filters.

Because of the importance of the L 11 bougie in differential filtration, the tests have been repeated with the particular sample of vesicular stomatitis virus at hand. Seven trials were made with the active material suspended in phosphate buffer at pH $= 7.5$ and at 8.5. At pH $= 7.5$, the virus passed through only one of three bougies but a portion of the same active material at pH $= 8.5$ traversed all of the four L 11 filters employed.

In the case of the virus of foot-and-mouth disease, a similar phenomenon was interpreted as evidence that the incitant is electropositive—a conclusion which was confirmed by the behavior of the virus on cataphoresis and by the determination of its isoelectric range at pH $=$ about 8. Although cataphoresis tests have not been

[13] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 685.

made with the virus of vesicular stomatitis, the indications are that it conforms with that of foot-and-mouth disease in respect to magnitude, charge, and isoelectric range.

Effect of Hydrogen Ion Concentration on Viability.

It has already been shown by Bedson and Maitland,[14] by Stockman and Minett,[15] and by us[16] that the virus of foot-and-mouth disease survives longest in a medium of which the hydrogen ion concentration is at pH = 7.5 to 7.6, and that viability is diminished considerably by slight variations above or below this narrow range. The same conditions apply to the virus of vesicular stomatitis. For example, the latter remained alive in phosphate buffer at pH = 7.2 and 7.5 to 7.6 for at least 52 hours at 37°C., but was inactive at pH = 6.8 and 8.0 at this time. After 100 hours, however, only the material at pH = 7.5 to 7.6 was active. The precise end-point was not determined.

Resistance to Chemicals.

The virus of foot-and-mouth disease is highly resistant to chemicals, such as alcohol, ether, chloroform, glycerol, and to many so called antiseptics, such as bichloride of mercury, cresol, phenol, etc.[14,15,17] We have pointed out that this remarkable resistance is due to the fact that the chemicals coagulate the proteins of the medium in which the virus is suspended and these in turn prevent direct contact of the virus with the reagents. If the periodic phenomenon attending such processes is considered and coagulation is prevented, the virus is made to come directly under the influence of the chemicals. Under these conditions, it is more sensitive to destruction by them than is staphylococcus. Moreover, the virus of foot-and-mouth disease is destroyed as rapidly as staphylococcus, or even more rapidly, by substances

[14] Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[15] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

[16] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 833, and the Bureau Report.[1]

[17] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815, and the Bureau Report.[1]

such as sodium hydroxide (1 to 2 per cent) or antiformin (1 per cent), which do not form coagula.

A similar series of tests was made with the virus of vesicular stomatitis in which we selected as an example of the narcotic solvents, 60 per cent alcohol, and of other coagulating substances, bichloride of mercury (1:1000), cresol (3 per cent), and phenol (1 per cent). Of the non-coagulating chemicals sodium hydroxide (2 per cent) was chosen.[12]

The virus was still active after 24 hours in 60 per cent alcohol. But if sodium hydroxide (1:5000), in which it can survive for at least a day, was added, and coagulation thus prevented,[17] it was killed within 1 minute. In bichloride of mercury, cresol, and phenol, in the dilutions mentioned, active materials remained viable for at least 6 hours. Tests for longer periods were not made. In sodium hydroxide (2 per cent) the virus was killed within 1 minute. An additional test revealed that 3 per cent cresol containing 1 per cent sodium hydroxide also inactivated it within 1 minute.

The virus remained active for at least $4\frac{1}{2}$ months in 50 per cent glycerol buffered at pH $= 7.5$, and kept at 4–6°C.

In so far as the resistance to chemicals is concerned, therefore, the virus of vesicular stomatitis resembles that of foot-and-mouth disease.[17]

Survival of the Virus Outside the Body.

It has been found[18] that in moist or palpably dried garden soil, the virus of foot-and-mouth disease survives for at least 25 days, and in hay for at least 1 month. Active materials derived from vesicular stomatitis also maintain their activity for a considerable time after leaving the body. Infected guinea pig vesicle coverings remained infectious for at least 31 days in garden soil kept at 4–6°C., or at 20°C., in a moist or in a palpably dried state.

Respiration of the Virus.

A study of the respiration of the virus of vesicular stomatitis was undertaken with the idea of obtaining information concerning its living character. The active agent could not, however, be separated in a pure state from respiring living tissues, nor could it maintain its

[18] Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry.

life under the conditions imposed by the experiment. Such was also the case with the virus of foot-and-mouth disease.[18]

Effect of Ultra-Violet Light on Viability.

In contrast to the results of the tests on respiration are those of the following experiments, done with the collaboration of Dr. F. L. Gates, of The Rockefeller Institute, on the effects of different wave-lengths and energies of monochromatic ultra-violet light. Since no similar tests were made with the virus of foot-and-mouth disease, no comparison of the two viruses can be offered in this respect. The experiments are reported, however, as adding suggestive evidence on the relation of the incitant of vesicular stomatitis to living bacteria. This evidence was obtained by comparing the behavior of the virus with that of *Staphylococcus aureus* under similar exposure to ultra-violet irradiation.[19]

Aspirated vesicular contents diluted 1:10 in buffered broth at pH = 7.4 were employed as virus and a thin suspension of *Staphylococcus aureus* from an 18 hour broth culture as control. The surface of thin layers of 2 per cent agar buffered at pH = 7.4 in small Petri dishes was washed with each of these materials respectively. After exposure, the virus-agar was cut within the limits of the area of light penetration, ground in a mortar, suspended in phosphate buffer at pH = 7.5, and of this about 0.4 cc. was injected intradermally into the posterior pads of guinea pigs. An additional control of unexposed virus-agar strips of similar size was also used in each test. After exposure, the agar, seeded with staphylococci, was incubated overnight at 37°C., and the number of colonies appearing in the exposed areas was compared with that in like areas from the unexposed portion of the plates.

At lambda = 2675 Ångström units, with a total energy of from 512 to 540 ergs per sq. mm., all the staphylococci were killed; at the same wave-length and from 256 to 270 ergs per sq. mm., 87 to 97 per cent of the organisms were killed. In respect to the virus subjected to the greater energy all of five guinea pigs were negative after inoculation, and in the second instance in which the lesser energy was used only one of four guinea pigs showed the experimental disease. All of five guinea pigs injected with unexposed virus-agar (controls) revealed typical lesions.

At lambda = 3022 Ångström units, with a total energy of 23,300 to 29,900 ergs per sq. mm., 97 to 100 per cent of the staphylooocci were killed. The exposed

[19] Olitsky, P. K., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 431.

virus-agar inoculated into four guinea pigs failed to infect, but the unexposed virus-agar (control) induced the experimental disease in all of four animals.

At lambda = 3126 Ångström units with a total energy of 60,400 ergs per sq. mm., there was no visible effect on the staphylococci. The exposed virus-agar proved active in three of four guinea pigs, and the unexposed similar material, as a control, induced the experimental disease in all of four animals.

It is thus evident that the transmissibility of the virus of vesicular stomatitis is lost on exposure to the same wave-lengths and energies of monochromatic ultra-violet light that are bactericidal. Furthermore, at the limits of destructive action of ultra-violet light, the reaction of the virus parallels that of the microorganism. Since adsorption of specific energies is one index of chemical character, these parallel reactions suggest that the substance of the virus is similar in character and chemical constitution to bacterial protoplasm.

<div align="center">DISCUSSION.</div>

In an earlier paper[1] reference was made to the similarity of the virus of vesicular stomatitis and of foot-and-mouth disease. Slight differences between them were ascribed to the variability of different strains. In the foregoing pages, additional evidence is presented to show their resemblance in several other reactions, physical, chemical, and biological. Furthermore, the clinical appearance of the diseases produced by the two viruses may be the same. Under field conditions and among cattle, the method employed heretofore in the United States for differential diagnosis has been to inject suspected material into a horse. If it reacted with typical vesicular lesions, a diagnosis of vesicular stomatitis was made; if it did not, the material was designated as having been derived from foot-and-mouth disease.[20]

There are, therefore, under consideration two viruses differing practically only in an absence of cross-immunity in recovered animals and in the resistance of the horse to one of them. In view of the non-cultivability of either virus, it is difficult to prove the precise relationship of one to the other. Yet, if a comparison be made with what exists among the types of foot-and-mouth disease virus itself or among known, cultivable bacteria, a close relationship between the two may be inferred. For example, there are at least two types of

[20] Mohler, J. R., *U. S. Dept. Agric., Dept. Circular 400*, 1926.

the active agent of foot-and-mouth disease, either of which shows no cross-immunity to the other in recovered animals. While all strains are generally active in cloven foot animals, there are some to which the guinea pig is resistant. Here, then, is a genus containing types which do not exhibit cross-immunity and show different pathogenic effects in a different species of animal. Among examples of similar behavior of cultivable microorganisms may be mentioned those dealt with in the recent studies of Tillett.[21] The rabbit which is susceptible to infection with Type I and Type II pneumococcus, is practically resistant to Type III. It is well known, moreover, that these different types do not show cross-immunity.

It appears, therefore, from the resemblance of the viruses of vesicular stomatitis and of foot-and-mouth disease, as demonstrated in this and in an earlier paper,[1] that their relationship is close. But this is an inference based on indirect evidence and is tentative until artificial cultivation of the viruses is obtained.

CONCLUSIONS.

A taxonomic study of the virus of vesicular stomatitis is presented along with evidence additional to that already reported[1] to show the similarity of this virus to that of foot-and-mouth disease. The connection of the two is discussed and the deduction drawn that their generic relationship is close. On the contrary, the differences between these two viruses and the herpetic are sufficiently marked to indicate a lack of generic connection among the three.

The results of a comparative study on the effects of particular wave-lengths and energies of monochromatic ultra-violet light on the virus and on *Staphylococcus aureus* reveal that the adsorption of specific energies by the two is parallel. Since the adsorption of specific energies is an index of chemical character, these experiments suggest that the virus is similar in character and chemical constitution to bacterial protoplasm.

[21] Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 1093.

THE INFLUENCE OF OVARIECTOMY ON THE SPONTANEOUS OCCURRENCE OF MAMMARY CARCINOMAS IN MICE.*

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Lathrop and Loeb (1), working on strains of mice with a high percentage of spontaneously developing tumors, have shown that the castration of female mice below the age of 6 months leads to a marked decrease in the tumor incidence. In a later report Loeb (2) was able to confirm and enlarge his previous results.

According to Loeb an ovarian hormone regulates those tissue changes which lead to the development of breast tumors in mice. The relationship between this hormone and the development of tumors is quantitatively graded. If the quantity of hormone acting on the breast tissue exceeds a certain limit, cancer appears as frequently in castrated as in non-castrated animals. This is the case when the mice are operated at 8 to 10 months of age. If an intermediate amount of hormone has been allowed to act on the mammary gland, that is, if castration has been performed at an age of 5 to 7 months, the cancer rate is noticeably diminished and the tumors appear at a later age period than in non-operated controls. If, finally, the quantity of the hormone has been restricted still further by operating on the animals at a still younger age, tumors either do not appear at all or only in exceptional cases. Loeb concludes that the hereditary factors which determine the development of breast carcinomas in mice need the cooperation of a definite quantity of ovarian hormone, if cancer is to develop.

As far as the writer is aware, the experiments of Loeb have not been repeated. This may be due to the fact that strains of mice with a

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* Presented before the nineteenth annual meeting of the American Association for Cancer Research.

high tumor incidence are rather difficult to obtain. Several years ago, Mr. Marsh, the biologist of this Institute, succeeded in evolving such a strain by selective crossing. Reports concerning the tumor incidence of this strain have been made on several occasions (3, 4). In eight inbred generations, covering a period of 5 years, the cancer rate and cancer age varied but little. Close to 94 per cent of all the breeding females that reached the cancer age developed breast carcinomas. Tumors appeared as early as 5 months and reached a maximum incidence at the age of 9 months.

It is necessary to use non-breeding females as controls for the castrated animals, since prevention of breeding decreases the percentage of tumors developing and increases the age at which tumors occur. Table I[1] indicates that of 42 non-breeding females living to tumor age, 33 developed spontaneous breast carcinoma. This corresponds to a tumor incidence of 78.5 per cent. In a previous experiment, 14 out of 18 non-breeding females developed tumors, which corresponds to an incidence of 77.7 per cent. The first tumors appeared later than in breeding females, namely at 10 to 11 months of age, while the highest tumor incidence occurred between 14 and 15 months of age.

Two series of experiments were made, but for reasons to be stated later only the second series is reported here in detail. In the first series groups of 20 to 30 female mice were castrated at 2, 3, 4, 5, and 6 months of age. The operation was performed by the abdominal route, consisting of the removal of the entire capsule containing the ovary, the Fallopian tube, and the tip of the uterus horn. Males were kept in the same cages as the operated females, but a pregnancy was never observed. An autopsy was performed on most animals that died spontaneously and on all animals that developed a tumor.

Surprisingly enough, it was found that about 15 per cent of the castrated animals showed one and in a few cases even two ovaries at the site of the previous operation. Upon microscopic examination some of these ovaries were in a state of degeneration or even calcified, others, however, showed follicles and corpora lutea. The possibility, that the ovaries had been incompletely removed, seemed very remote. It was, therefore, concluded that a regeneration of ovarial tissue had taken place. This was amply confirmed by a paper by Davenport (5) that appeared later.

[1] Compiled from the statistics kindly furnished to us by Mr. Marsh.

This author made a detailed study of the conditions under which ovaries regenerate in mice. Regeneration is less apt to occur when the capsule, tube, and part of the fat body surrounding the ovary are removed than when the ovary alone is removed by cutting its stalk and picking it out of the capsule. With the former method of re-

TABLE I.

Monthly Expectancy of Tumor Incidence in Non-Breeding Female Mice (Strain 3).

Based on 42 mice living to tumor age, of which 33 developed tumors. Incidence: 78.5 per cent. Females isolated at 1 month of age, one in each cage. Remained isolated throughout life. The food consisted of dog bread.

Age	No. of tumor mice	Per cent of 42 tumored during each month	Total per cent tumor mice at end of each month
mos.			
1–10	0	0	0
11	2	4.76	4.76
12	2	4.76	9.52
12½	2	4.76	14.28
13	1	2.38	16.66
13½	1	2.38	19.04
14	4	9.52	28.56
14½	1	2.38	30.94
15	6	14.28	45.22
15½	1	2.38	47.60
16	2	4.76	52.36
18	2	4.76	57.12
19	2	4.76	61.88
20	5	11.90	73.78
20½	1	2.38	76.16
21	1	2.38	78.54
	33	78 54	

The 9 mice without mammary tumors lived 7 to 18 months, average 12.5 months. One of these mice developed a spindle cell sarcoma. Reckoned as a clean mouse above.

moval, regeneration took place in 18 per cent of the cases, but when the ovary alone was removed, regeneration was observed in 64 per cent of the operated animals. Regeneration may take place as early as 1 and as late as 6 months after the operation. The age had no demonstrable effect upon the chance of regeneration, except when the mice

were very young. In several cases two ovaries regenerated in the place of one or the new ovary arose at a point $\frac{1}{2}$ cm. distant from the position of the former ovary.

There seems no doubt that the ovary of the mouse may regenerate from the peritoneum under favorable conditions. Prior to Davenport, Castle and Phillips (6) observed ovarian regeneration in 11 and pregnancy in 3 out of 141 operated guinea pigs.

Interesting as it may seem that ovaries regenerate in mice, it was a rather unfortunate occurrence from the point of view from which these experiments were undertaken. Animals that showed ovaries upon postmortem examination, whether they were normal or tumor mice, had to be ruled out. When these were omitted, there remained 49 animals that were still alive at an age of 19 months. 44 of these mice remained free from tumors until they died of old age or of intercurrent diseases. 5 animals out of these 49 developed tumors, 1 at $18\frac{1}{2}$ months, 1 at 19 months, 2 at 20 months, and 1 at 21 months. This gives a tumor incidence of 10 per cent, as compared with 78.5 per cent for the non-breeding controls. There seems no doubt that ovariectomy performed between 2 to 6 months of age reduces the tumor incidence and increases the tumor age.

In the second series of experiments the control of the experimental conditions was very satisfactory. The underlying idea was to see if the occurrence of tumors could be prevented altogether by operating on the animals at a very young age. 100 mice were castrated between 15 and 22 days of age, that is, immediately after weaning. The ovaries were exposed by the lumbar route and the entire fat body surrounding the ovary together with a portion of the uterus horn were removed. Beginning with 6 months, the mice were examined for tumors regularly once a week throughout their life time. Great care was taken to autopsy as many mice as possible.

In no case did the postmortem examination reveal the presence of ovarian tissue. However, from the experience gained in the first series of experiments it seemed unwise to rely on the autopsy alone. The absence of ovaries at the time of death was not considered proof enough that no regeneration had taken place, since most of the animals were very old when they were examined. A regenerated ovary might have disappeared again through absorption. Use was made of the vaginal smear method of Stockard and Papanicolaou (7), which indi-

cates in a definite way whether or not ovarian activity is present. In the mouse the estrus cycle lasts from 4 to 6 days. At the height of the estrus the vaginal smear is characterized by the presence of large cornified cells without a nucleus and by the complete absence of leucocytes. In the resting stage of the estrus cycle a large number of leucocytes are present and some small, nucleated epithelial cells, while the characteristic cornified cells are missing. For 1 consecutive

TABLE II.

Absence of Spontaneous Mammary Carcinomas in Mice Ovariectomized between 15 and 22 Days of Age.

Most of the operated mice belonged to Strain 3, which has a tumor incidence of 78.5 per cent. A few mice were from Hybrid Strain 1, which has a tumor incidence of 50 per cent.

Age	No. of mice alive	Remarks
mos.		
6	94	
10	86	
12	84	
14	79	
16	73	1 killed at 15 mos. Spindle cell sarcoma of fore leg.
		1 killed at 15½ mos. Spindle cell sarcoma of tail.
18	72	
20	60	
22	37	1 killed at 21 mos. Spindle cell sarcoma of abdominal wall.
24	14	
26	5	
28	1	

week vaginal smears were made on each mouse 2, 4, and 6 months after the operation. Only one mouse with a positive vaginal smear was picked out in this way. This animal through an oversight proved not to have been operated upon. The fact that the vaginal smears were all negative makes it certain that the mice of this series were entirely free from ovarian activity throughout their life time.

The result was that none of these mice developed a spontaneous

adenocarcinoma of the breast.   Table II contains a detailed record of this series of experiments.   From 86 mice that were still alive at 10 months of age, that is, at the time that the first tumors appeared in the non-breeding controls, 60 mice reached an age of 20 months.   At 20 months of age 73.8 per cent of the control mice had developed tumors, while none of the operated animals had shown a breast tumor so far.   At 21 months the last control mouse had died, while a larger number of operated mice continued to live up to 24 months and more. The chance to develop a tumor was, therefore, greater in the operated mice than in the control mice.   Nevertheless, all the operated animals remained free from breast carcinomas.   This remarkable effect of the ovarian hormone on the development of spontaneous breast tumors in mice seems to be a specific one, since the absence of the ovaries did not prevent the occurrence of other types of tumors.   It will be noted in Table II that 3 of the castrated mice developed spindle cell sarcomas.   A low incidence of spontaneous sarcomas has been observed previously in this strain of mice and is, therefore, nothing abnormal.   The conclusion seems justified that the lack of breast tumors in spayed mice is not due to some indirect effect of castration. The body changes produced by castration are not such that they lead to a resistance against spontaneous tumors in general.   Only the tumors arising from the mammary gland, a tissue that is directly influenced by the sex hormone, are prevented from developing. Another point of interest is that the prevention of breeding prolongs the life time of the mouse and that mice spayed at an early age live still longer than the non-breeding controls.

<div align="center">DISCUSSION.</div>

Loeb interpreted his results on the basis of the interrelation between the ovary and the mammary gland.   Since that time our knowledge of the ovarian function has been increased considerably, chiefly through the isolation of a potent ovarian hormone from the liquor folliculi by Allen and Doisy (8).   If the ovaries are removed before sexual maturity is reached, the mammary tissue remains in an undeveloped state throughout the life of the animal, similar to the undifferentiated state of the breast tissue of the male animal.   Our experiments indicate that a malignant change does not occur in this

undifferentiated tissue. Nevertheless, these animals must be regarded as potential tumor bearers, since they have inherited an organ disposition to develop tumors from previous generations. But the hereditary organ disposition alone does not lead to carcinoma of the mammary gland. The cooperation of the ovarian hormone is necessary, first, to cause the full development of the breast and second, to induce a periodic stimulation of the mammary tissue. During the life of the adult female the estrus cycle, which lasts from 4 to 6 days, provides a periodic stimulation of the breast tissue. One can calculate that a mouse has about 70 to 80 estrus cycles during life. If castration has been performed at 2 to 5 months of age, that is, after 3 to 20 estrus cycles have taken place, the occurrence of tumors is greatly reduced but not entirely suppressed. Castration after 6 to 7 months, that is, after 30 periodic enlargements of the mammary gland, has no decided influence on the tumor incidence. The tumors appear at the same rate as in the animals that had intact ovaries throughout their life. The point of interest is that a relatively short period of functional activity of the mammary gland leads to those tissue changes that result later on in the development of carcinoma. Equally striking is the long latent period between the ovarian stimulus that leads to cancer and the onset of the cancerous change itself.

When the tumor is once established, the ovarian hormone seems to be without influence on the rate of growth of the tumor. This is shown by the fact that spontaneous tumors in previously castrated animals do not grow less rapidly than tumors developing in normal animals. Even a surplus of ovarian hormone does not increase the rate of growth of mammary carcinoma, as may be judged from experiments that have been made with the ovarian hormone of Allen and Doisy. This hormone, when injected into spayed animals, produces the typical estrus changes in the genital organs and causes an enlargement of the milk ducts of the breast with proliferation of the glandular elements. It seemed of interest to investigate, whether this hormone would also have a growth-promoting effect on the adenocarcinoma which arises from the normal glandular elements of the breast. 50 normal females with either spontaneous or transplanted breast carcinoma received 4 to 10 hormone injections at 5 day intervals. The potency of the hormone had previously been tested on spayed animals.

However, no definite influence of this surplus of ovarian hormone on the rapidity of growth of the tumors could be observed.

It is a well known fact that the males belonging to inbred strains of mice with a high tumor incidence, do not show mammary carcinoma. Even though the males transmit the hereditary factors, they remain free from tumors, because the mammary tissue remains undeveloped in analogy to the female mice castrated before puberty, which also remained free from tumors. Loeb tried to produce breast tumors in males by transplanting ovaries into them, but obtained negative results. The author also obtained negative results by this procedure. Males belonging to the high tumor strain were castrated at an early age. When they were 2 and 6 months old, two ovaries of adult females were transplanted into the peritoneal cavity of each mouse. Most of the animals reached an old age, but breast tumors were not observed. It was also tried to produce breast tumors in males by the injection of the ovarian hormone. The hormone was prepared from the liquor folliculi of hogs according to the original prescription of Allen and Doisy (8). The chief difficulty was that at that time the only suitable solvent for the hormone was oil. The injections could not be extended over 10 weeks without causing infiltrations and general impairment of the health of the animals. Many months after the injections had been stopped, unabsorbed oil was still present in the subcutaneous tissue. A positive result could only be expected, if the injections could be continued throughout life. One of the animals developed a sarcoma adjacent to an oil cyst, the other animals remained free from tumors. Recently it has been possible to prepare the hormone in a water-soluble form. The experiments on male animals are being repeated, since it would be of considerable importance to produce breast tumors in male animals experimentally.

SUMMARY AND CONCLUSIONS.

1. Castration of female mice between 15 and 22 days of age entirely prevented the occurrence of spontaneous adenocarcinoma of the breast, while the non-breeding control mice of the same strain showed a tumor incidence of 78.5 per cent. 3 of the spayed animals developed a spontaneous spindle cell sarcoma. This indicates that the influence

of the ovary on the development of breast carcinomas is a specific one, since ovariectomy does not lead to a resistance against other types of spontaneous tumors.

2. Castration between 2 and 6 months of age led to a marked reduction of the tumor incidence, but did not entirely prevent the occurrence of mammary tumors.

3. It is concluded that the spontaneous mammary carcinoma of the mouse is due to a hereditary organ disposition, which remains latent in the absence of ovarian function, but which becomes manifest when a certain amount of ovarian hormone, corresponding to 5 to 30 estrus cycles, has acted on the breast tissue.

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FURTHER OBSERVATIONS CONCERNING GROWTH REQUIREMENTS OF HEMOPHILIC BACILLI.

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Hemophilic bacilli form a heterogeneous group of bacteria. Observations concerning their growth requirements and the hemolysis caused by some strains have been reported. By means of these observations a subdivision of this group of organisms has become possible.

Pfeiffer (1), 1893, first described *B. influenzæ*. He was unable to cultivate the bacillus in the absence of blood pigment. Grassberger (2), 1898, reported that Pfeiffer's bacillus in symbiosis with *Staphylococcus aureus* was able to multiply on media containing hematin. Olsen (3), 1920, showed that growth of *B. influenzæ* occurred only on media giving a positive peroxidase reaction. Fildes (4), 1920, obtained satisfactory growth of Pfeiffer's bacillus on media enriched by means of a peptic digest of blood. Later, in 1921, he also showed (5) that in this digest each of two factors was essential for the growth of the organism; one was present in the clear supernatant fluid, the other occurred in the brown hematin deposit. Avery and Thjötta (6, 7), 1921, in a series of papers, reported that two accessory factors are necessary for the cultivation of influenza bacilli. These factors were designated as X and V. The V substance is relatively heat-labile and is found in blood, in cultures of many bacteria, and in yeast and vegetable cells. The X substance is heat-stable, gives a positive peroxidase test, and is found in blood and raw potatoes. Rivers and Poole (8), 1921, showed that two accessory factors are essential for the growth of influenza bacilli; one is present in filter-sterilized yeast extract, the other in autoclaved extract of blood. Rivers (9, 10), 1922, reported that Friedberger's *B. hemoglobinophilus canis* requires only the X accessory factor, and that from patients 2 strains of bacilli had been obtained that require only the V accessory factor. The strains requiring only V factor were designated *B. parainfluenzæ*. Rivers and Bayne-Jones (11), 1923, obtained from throats of cats 6 strains of influenza-like bacilli that required only the V factor.

Pritchett and Stillman (12), 1919, described a Gram-negative, aerobic, non-motile, hemophilic bacillus and designated it as Bacillus "X." Stillman and Bourn (13), 1920, reported further observations concerning the characteristics of Bacillus "X." Rivers (14), 1921, suggested that these organisms be called hemolytic influenza bacilli. Rivers (10), 1922, on testing a hemolytic strain found that

it required both X and V growth accessory factors.   Fildes (15), 1924, reported that 13 of 14 strains of hemolytic influenza bacilli required only the V factor. The remaining strain, however, required both X and V.   The former strains grew on ordinary media in symbiosis with staphylococci, and also with *B. hemoglobinophilus canis* on media containing the X factor.   This fact indicated to Fildes that *B. hemoglobinophilus canis* synthesizes the V factor.

Particular interest in the hemophilic bacilli was again aroused by the occurrence in New York during the spring of 1926 of a respiratory infection resembling mild influenza.   Attempts were made to determine (1) how frequently hemophilic bacilli occur in the upper respiratory tract of patients not necessarily suffering from influenza, and (2) what variations in growth requirements exist among strains of these bacilli obtained from such sources.   The results of this investigation are dealt with in the present paper.

Methods and Materials.

Autoclaved meat infusion broth was used as a basic medium.   Filter-sterilized yeast extract, prepared in the manner described by Avery and Thjtta, supplied the V factor.   0.5 cc. of the extract was added to 5 cc. of broth.   A solution of hematin, prepared in the manner described previously (11), supplied the X factor. Dilutions, 1 to 10,000, of this solution gave positive reactions for the presence of peroxidase.   When the presence of the X factor was desired in the medium, 1 cc. of the hematin solution was added to each 100 cc. of broth.   The broth or yeast extract alone never gave positive peroxidase tests.

Bacilli used in this work were obtained from the throats of patients by means of cultures on fresh 2 per cent rabbit blood agar plates.   Hemolytic and non-hemolytic Gram-negative bacilli isolated from these plates were carried in stock on blood agar slants.   A strain from the spinal fluid of a patient with influenzal meningitis and 1 from a pneumonic lung were also studied.

The bacilli to be tested were removed from blood agar slants by means of a platinum loop and transferred to tubes of broth containing yeast extract, hematin, and yeast extract and hematin respectively.   In the case of non-hemolytic strains that grew only in broth containing both factors (X and V), and of hemolytic strains that required the addition of only the V factor, macroscopic evidence of growth was considered sufficient and no subcultures on solid media were made. Other strains, however, were tested more completely in regard to their growth requirements.

EXPERIMENTAL.

From 46 patients, 34 strains of so called hemophilic bacilli were isolated.   In Table I the sources from which they were obtained and

the frequency with which they occurred in certain groups of cases are indicated. In some of the groups too few cases were studied to permit of a comparison between the groups. Omitting the 4 cases of influenza, however, 42 cases of other diseases were examined and the results are interesting and significant. From these 42 cases, 18 strains of non-hemolytic bacilli (43 per cent) and 11 strains of hemolytic ones (26 per cent) were obtained. Although the patients did not have influenza, 29 of them (69 per cent) harbored hemophilic bacilli in their throats. These findings are in accord with those of other workers who have shown that hemophilic bacilli are likely to be encountered very frequently during periods of epidemic respiratory infections.

TABLE I.

Sources of the Hemophilic Bacilli Studied.

Type of case	Number examined	Number of non-hemolytic strains isolated	Number of hemolytic strains isolated
Chicken-pox	27	8	7
Measles	9	6	3
Influenza	4	1	4
Miscellaneous	6	4	1
Total............	46	19	15

The 34 strains of bacilli were tested in the manner described above to determine their need of growth accessory factors, V and X. The results are summarized in Table II.

From Table II it is obvious that 17 of the non-hemolytic strains required both growth accessory factors (X and V). They were similar in every respect to organisms usually accepted as influenza bacilli (Pfeiffer). There were 2 non-hemolytic strains, however, that grew well when yeast extract (V) alone was added to the broth. These organisms were carried through 7 successive transplants in this medium. Subcultures were pure. In broth alone or in broth plus hematin, with 0.25 cc. amounts for transfers, no viable bacilli were found by means of subcultures on blood agar after the 2nd transfer. Neither strain produced indole. Both reduced nitrates to nitrites.

Each grew diffusely in liquid media. These bacilli corresponded to Rivers' *B. parainfluenzæ*.

There were 10 hemolytic strains that grew well in broth to which only yeast extract (V) was added. These bacilli were fairly uniform in type. Cultures of them in liquid media usually exhibited flocculi similar to the ones seen in streptococcus cultures. On blood agar plates, colonies of the bacilli were surrounded by large zones of hemolysed red blood cells, were slightly opaque, and were often firm enough to be pushed about intact on the surface of the medium.

TABLE II.

Summary of Results Obtained Concerning the Growth Requirements of the Hemophilic Bacilli.

Type and number of strains	Broth + yeast extract	Broth + hematin	Broth + yeast extract + hematin	2 per cent rabbit blood agar
17 non-hemolytic	−	−	+	+
2 non-hemolytic	+	−	+	+
10 hemolytic	+	−	+	+
3 hemolytic	−	−	+	+
2 hemolytic	−	−	−	+
1 *B. influenzæ*, control	−	−	+	+
1 *B. parainfluenzæ*, control (original Rivers strain)	+	−	+	+
1 *B. hemoglobinophilus canis*, control	−	+	+	+

The + sign indicates visible growth. The − sign indicates no growth. Yeast extract supplied the V factor; hematin, the X factor.

Upon microscopic examination the bacilli were found to be exceedingly pleomorphic and usually larger than Pfeiffer's bacillus. In order to maintain stock cultures on blood agar slants it was necessary to make transfers more frequently than at weekly intervals.

Three hemolytic strains required both X and V growth accessory factors. They were carried through 7 successive transplants in broth containing yeast extract and hematin. In the later transfers, successful transplants were obtained by means of a platinum loop. In broth containing yeast extract or hematin alone no viable bacilli were present after the 3rd or 4th transfer. 2 other hemolytic strains that grew

well in rabbit blood broth were not viable after 3 or 4 transfers in broth containing both yeast extract and hematin. It is possible, however, that after a longer period of cultivation on artificial media, results with these 2 strains might have been similar to those obtained with the 3 strains described above. On blood agar plates colonies of these 5 strains were surrounded by very narrow zones of hemolysed red blood cells. They were small, transparent, moist, and soft. In fluid media the bacilli always grew diffusely. All 5 strains reduced nitrates to nitrites. One produced indole. Upon microscopic examination these organisms resembled Pfeiffer's bacillus. Weekly transplants of the organisms on blood agar were found to be sufficient for maintenance of stock cultures.

From the results of the experiments already described, it is evident that some hemophilic bacilli hemolyse red blood cells, while others do not, that some require both X and V growth accessory factors, while others require either X alone or only V. Fildes showed that organisms requiring only V, or both X and V, multiplied in symbiosis with *B. hemoglobinophilus canis* on media containing only X. From this observation he concluded that the latter organism synthesized V and supplied it to the bacilli needing it for growth. Since some hemophilic bacilli require the addition of only V to the media used for their cultivation, and since it has been shown that many bacteria capable of growing on ordinary media, *e.g.* staphylococci, give a positive peroxidase reaction, experiments were performed to determine (1) if the hemophilic bacilli capable of growing on media to which only V is added give a positive peroxidase test, and (2) if they are able to provide the X factor to bacteria needing it for growth. The results of these experiments will be described below.

In the experiments to determine whether the bacilli requiring the addition of only V to the media give a positive test for peroxidase, the following strains were employed: Rivers' original *B. parainfluenzæ*, 2 non-hemolytic strains which were described above and which will be spoken of as *B. parainfluenzæ* Nos. 2 and 3, and 1 hemolytic strain. The 4 strains of bacilli were grown in broth to which yeast extract had been added. Cultures 24 hours old were used in every instance. A portion of each culture was saved for tests. The remaining portion was centrifuged and the sedimented bacilli were resuspended in salt solution. This procedure was repeated twice. A portion of the original culture, the last supernatant salt solution, and the washed bacilli suspended in salt solution were

tested for the presence of peroxidase in the usual way by means of benzidine. The suspension of bacilli was much denser in the salt solution than in the original broth cultures. The results of the experiments are shown in Table III.

The results of the experiments summarized in Table III indicate that the hemophilic bacilli requiring the addition of only yeast extract (V) to the media give a positive test for peroxidase (X) in spite of the fact that the medium in which they are grown gives a negative one.

B. hemoglobinophilus canis requires the addition of X to its media, yet apparently produces V.   *B. parainfluenzæ* and some of the hemolytic bacilli require the addition of V to their media, but give a positive test for peroxidase (X).   In view of these facts it seemed of in-

TABLE III.

Results of Benzidine Tests for Presence of Peroxidase.

Strains tested	Original culture	Supernatant salt solution	Washed bacilli
B. parainfluenzæ, No. 1	±	—	+
B. parainfluenzæ, No. 2	—	—	±
B. parainfluenzæ, No. 3	—	—	±
Hemolytic bacillus	—	—	±

The ± sign indicates a faintly positive reaction.   The + sign indicates a positive reaction.   The — sign indicates no reaction.

terest to ascertain whether the organisms requiring the addition of X alone and the ones needing the addition of only V are capable of supplementing the growth accessory factors for each other when seeded together in media to which neither X nor V has been added. The manner in which this question was investigated is described below.

Autoclaved meat infusion broth and meat infusion agar to which no growth accessory factors had been added were the media employed.   *B. hemoglobinophilus canis* requiring the addition of X alone, *B. parainfluenzæ*, Nos. 1, 2, and 3, needing the addition of only V, and a hemolytic bacillus also requiring the addition of only V were the organisms studied.   When a liquid medium was used transfers were effected by means of pipettes, 0.25 cc. being the size of the inoculum. Cultures were incubated 72 hours between transplants.   Subcultures on blood agar plates were employed to determine the viability and the types of the bacilli in the different tubes.   The ability of the bacilli to grow alone or together under the conditions mentioned is indicated in Tables IV and V.

TABLE IV.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli in Meat Infusion Broth.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	+	?	−				
2. *B. parainfluenzæ*, No. 1	X	+	?	?	−			
3. *B. parainfluenzæ*, No. 2	X	+	−					
4. *B. parainfluenzæ*, No. 3	X	+	?	−				
5. Hemolytic bacillus	X	+	?	−				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The − sign indicates no growth when subcultures were made on blood agar. The + sign indicates visible growth of bacilli of the type or types with which the series was started.

TABLE V.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli on Meat Infusion Agar.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	?	−					
2. *B. parainfluenzæ*, No. 1	X	?	−					
3. *B. parainfluenzæ*, No. 2	X	?	−					
4. *B. parainfluenzæ*, No. 3	X	?	−					
5. Hemolytic bacillus	X	?	?	−				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The − sign indicates no growth when subcultures were made on blood agar. The + sign indicates a visible growth of bacilli of the type or types with which the series was started.

From the results shown in Tables IV and V, it is obvious that bacilli requiring the addition of X alone and those requiring the addition of only V to their media grow well in symbiosis on media to which neither X nor V has been added. This fact indicates that these organisms are capable of supplementing the growth accessory factors for each other when grown together under the conditions of the experiments.

<div align="center">DISCUSSION.</div>

The results of experiments reported in the first part of the paper support the findings previously recorded by Rivers and Fildes concerning the growth requirements of non-hemolytic and hemolytic hemophilic bacilli. Furthermore, these results indicate that a subdivision of this group of bacilli based upon their growth requirements is not only possible but also practicable. The majority of the non-hemolytic strains require the addition of both X and V to their media, while a few need the addition of only V. On the other hand, most hemolytic strains require the addition of only V to their media, while some need the addition of both V and X. It is true that 2 hemolytic strains were encountered that were susceptible to cultivation in blood broth but not in broth containing yeast extract and hematin. In view, however, of their recent isolation and of the difficulty experienced with 3 similar strains that required both factors, it seems justifiable to consider the 5 strains as members of one group, which, in its general characteristics, is more closely related to Pfeiffer's bacillus than to the other hemolytic bacilli under discussion.

The results of the investigation reported in the latter part of the paper apparently indicate that strains of hemophilic bacilli requiring the addition of only V or X to their media are capable of supplying, or acting as, X or V respectively to an extent sufficient for the needs of other bacilli that require the addition of these factors to their media. This fact supports the view that organisms which do not require the addition of either growth accessory factor to their media only multiply on such media because they are capable of synthesizing these factors.

Discussions have arisen in regard to the number of bacilli that should be included in the hemophilic group. Some workers contend

that this group of organisms should not be regarded as hemophilic, inasmuch as all of its members under certain conditions are capable of growth in the absence of blood. There is evidence, also, that in the past similar organisms have been classified under different headings, *e.g.*, Koch-Weeks' bacillus and Pfeiffer's bacillus are identical. Because of the variety of organisms that have been placed in this group any kind of a classification will have some objectionable features. Knowledge, however, recently acquired concerning the growth requirements of the hemophilic bacilli and the ability of some of them to hemolyse red blood cells enables one to outline a relatively simple classification of this heterogeneous group of organisms. Such a classification is presented below. The growth requirements, X and V, of a few bacilli placed in the hemophilic group have not been determined. When this has been accomplished these organisms can then be given their proper place in the classification presented.

Classification of Hemophilic Bacilli.

A. Requiring the addition of V and X to the media.
  B. influenzæ (Hemophilus influenzæ).
  1. Non-hemolytic.
  2. Hemolytic.
B. Requiring the addition of only V to the media.
  B. parainfluenzæ (Hemophilus parainfluenzæ).
  1. Non-hemolytic.
  2. Hemolytic.
C. Requiring the addition of only X to the media.
  B. hemoglobinophilus canis (Hemophilus canis).
D. Requiring the addition of neither X nor V to the media.
  1. *B. pertussis (Hemophilus pertussis).*

SUMMARY.

1. 19 strains of non-hemolytic hemophilic bacilli were studied. 17 required the addition of V and X growth accessory factors to their media, 2 required the addition of only V.

2. Of 15 strains of hemolytic hemophilic bacilli examined, 10 were found to require the addition of only V to their media, 3 the addition of V and X in the form of yeast extract and hematin, and 2 the addition of accessory growth factors in the form of blood.

3. In media to which neither V nor X had been added true symbiosis was found to occur on growing *B. hemoglobinophilus canis*, requiring the addition of X, with *B. parainfluenzæ*, requiring the addition of V, or with hemolytic strains of bacilli, requiring the addition of only V.

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A SIMPLE METHOD FOR THE ISOLATION OF PURE CULTURES FROM SINGLE BACTERIAL CELLS.

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The use of "pure line" strains of bacteria is required in many problems encountered in bacteriological research. A number of methods have been devised for obtaining cultures which represent the progeny derived from a single cell. Most of these methods possess at least one of the following disadvantages: (1) intricate apparatus, (2) complicated technique, (3) loss of time, due to the frequent failure of a single cell to grow when transplanted.

During studies on variants occurring in streptococcus cultures, a simple method was devised by which pure line strains can be obtained with comparative ease. This method represents a modification of the Hansen[1] method for the isolation of pure cultures of yeast. Although Hansen's method has been employed extensively in the fermentation industries for a number of years, its most advantageous features have not been incorporated in any of the previous bacteriological methods. The essential features of the method as adapted to the isolation of single cell cultures of bacteria are described below.

Apparatus and Materials.

Cover-Glass.—A large 43 × 50 mm. cover-glass, 0.13 mm. thick, is ruled into squares of 4 sq. mm. area by etching with a fine needle and hydrofluoric acid through paraffin. The system of numbering the squares is illustrated in Fig. 1. *Ring.*—The cover-glass is supported on a metal ring. Convenient dimensions proved to be 35 mm. diameter, 3 mm. height and 1 mm. thickness. The ring can be cut

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[1] Hansen, E. C., *Meddelelser Carlsberg Lab.*, 1886, ii, No. 4, 152.

from brass tubing. The metal side arms are attached for convenience in handling. *Glass Slide.*—The chamber is formed by attaching the ring to a large slide 50 × 75 × 1 mm. The assembled apparatus is shown in Fig. 2.

Inoculating Spatula.—The spatula used for inoculation is formed from a glass rod 4 mm. in diameter. One end is drawn out in a flame to 2 mm. in diameter, and the tip flattened and bent at a convenient angle.

Culture Medium.—Any clear solid medium suitable for the cultivation of the test organism may be used provided it is made as free as possible from all extraneous particulate matter. Freedom from particles in the culture medium is essential to the success of the method.

Technique.

All glassware should be scrupulously clean before use. Rings, glass slides and cover-slips are sterilized in separate Petri dishes by dry heat. Aseptic precautions are followed throughout the technique. The assembling of the different parts is done under the protection of the lifted Petri dish cover. The metal ring is dipped in hot paraffin and then placed in contact with the unetched side of the cover-slip. The paraffin is allowed to harden. The surface of the cover-glass within the ring is flooded with melted agar sufficient to cover the entire area. In order that the film formed may be the thinnest possible, a warm pipette is held at right angles to the surface and all the recoverable agar is withdrawn and discarded. After this delicate film of agar has hardened it is inoculated as follows: A loopful of a young broth culture is transferred to 5 cc. of infusion broth and thoroughly mixed. The sterile glass spatula is dipped into the dilute bacterial suspension and the excess drained off against the side of the tube. The spatula carrying the minimum inoculum is lightly stroked over the entire surface of the film. The inoculated cover-glass and attached ring are inverted and sealed to the large glass slide which has been previously ringed with paraffin.

The preparation is then examined under the microscope with a high dry objective. After a thorough search, squares containing only one organism are selected and the location of each plotted on a diagram with reference to the index number. The location of the

cell within the selected square is also recorded on the diagram. The orientation is further facilitated by the use of a mechanical stage. Usually six or seven squares, each containing a single organism, are plotted to insure against any failure of growth.
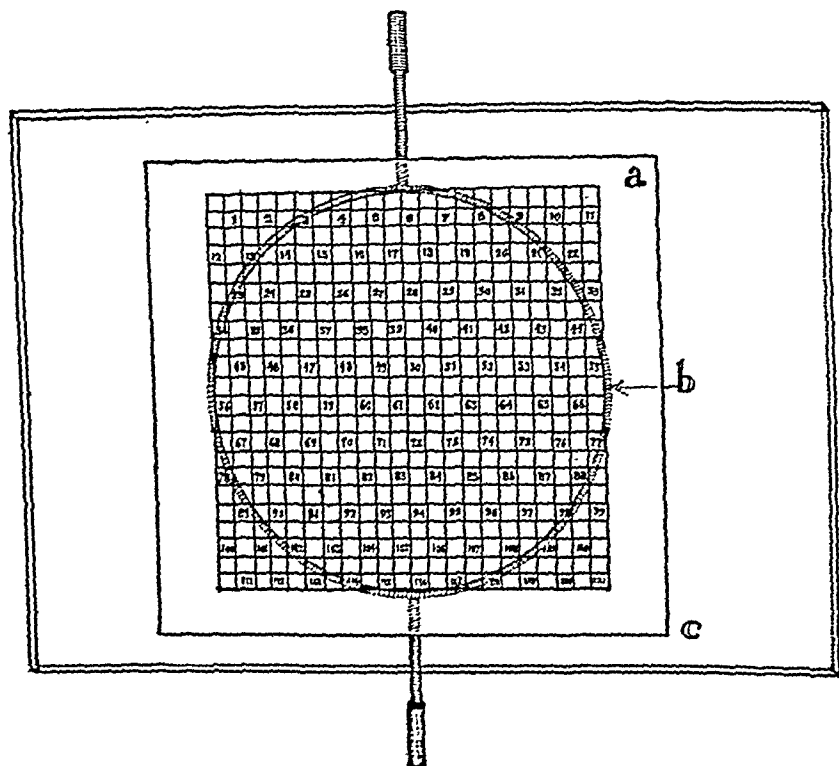
FIG. 1. Top view of apparatus. (*a*) Cover-glass; note that all squares not numbered are adjacent to numbered ones. (*b*) Brass ring and side arms. (*c*) Glass slide.

The entire apparatus can now be removed from the microscope stage and incubated at 37°C. until colony formation is well developed. At the end of the incubation period the length of which is determined by the growth rate of the particular organism, the preparation is again examined microscopically. With the aid of the diagram and the stage readings, the fields previously selected are located and the

individual square is again searched to make certain that it contains only a single colony. As each individual square is checked, its position on the cover-glass is indicated by an ink dot made with a fine pointed pen while the preparation is under low power magnification. The ring carrying the cover-glass is then removed and by means of a fixed reading lens the colony indicated by the dot is transferred with a fine platinum needle to a suitable culture medium.

The principal sources of error in the method are practically eliminated by the selection of cover-glasses of the proper thickness and by the use of a culture medium which is wholly translucent and free as possible from all microscopic particles. If the agar is spread in a very thin film, little difficulty is experienced in the selection of single bacterial cells, when the illumination is adjusted. The moisture of
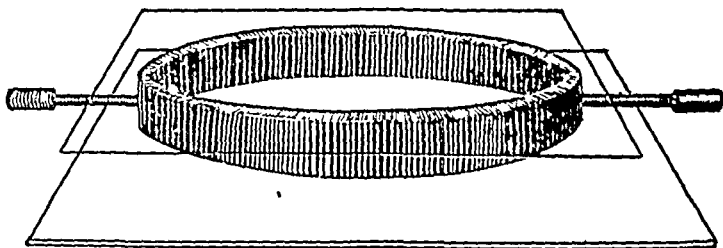
FIG. 2. Side view of apparatus.

the shallow chamber is maintained by the agar medium, which makes it possible to omit the excess fluid usually added in moist chamber preparations The excess moisture is not only unnecessary but is undesirable since the coalescence of drops of condensed water befog the field of vision and tend to cause the migration of bacterial cells.

COMMENT.

The procedure as outlined has been used in the study of streptococci and pneumococci but it is equally adapted to the isolation of other aerobic organisms and by slight modification might be made useful in the study of anaerobic types.

The method is simple in operation and no special technique is required to obtain the results desired. That several selected colonies can be isolated from the same preparation is most advantageous,

since the chance of successful transplant is thereby correspondingly increased. Moreover, the fact that a number of different colonies can be kept under observation on the same preparation, makes the method particularly adaptable to studies on variation in colony structure. In a successful preparation, it is often possible to obtain a dozen colonies, each of which is known to be derived from a single cell. This is a distinct advantage in the study of the origin of variants which differ in colony structure.

SUMMARY.

A method is described for the isolation of pure cultures from single bacterial cells, which is simple in operation and which requires no complicated apparatus. In addition to its simplicity, the method possesses the further advantage that several selected colonies can be isolated from the same preparation.

THE CULTIVATION OF LENS EPITHELIUM IN VITRO.*

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PLATE 30.

In the course of an investigation into the nature of the nutrition
of the crystalline lens and the cause of cataract, it was proposed by
the author that an attempt be made to cultivate the cells of the lens
in vitro. Such a strain of tissue might be used for controlled
experiments.

Since the discovery by Harrison (1) in 1907 that cells could be
caused to migrate and divide outside the body, various investigators
have used the method for the study of morphological and physiologi-
cal problems.

Carrel (2) developed an ingenious technique by which permanent pure strains
of fibroblasts could be isolated and cultivated *in vitro.* He established the pro-
cedures by which the method of tissue cultivation is becoming adapted to its
main object, the study of the fundamental problems of physiology and pathology.
The early technique consisting of placing a fragment of tissue in a hanging drop
of culture medium, did not allow an accurate analysis of the action of a tissue upon
other tissues and upon the humors. The cells were subjected to complex influ-
ences, such as those of necrotic cells of their own type, of living and dead cells
of other types, and of a medium which deteriorated spontaneously in a short
time (3).

Advances have been made during the last few years, chiefly through the em-
bryonic extract-plasma clot method of Carrel by which those difficulties and

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objections are overcome, and have contributed markedly to the progress of an important method of investigation, primarily by the isolation of pure strains of cells (4), and by the development of procedures for measuring the rate of growth and for studying the functional activity of the tissues (5, 6).

Fischer (7) isolated an unmixed strain of epithelium from the anlage of the ·chick iris in 1922. Ebeling (8) produced cultures of the thyroid epithelium in 1925. It has been possible to carry these cultures on for long periods of time. It has been proved that the cells remain true to type, that they do not dedifferentiate, and that they produce their typical substances *in vitro*.

In the experiments about to be reported the method and technique of Carrel (2–6), were used.[1]

Experiment Demonstrating the Metamorphosis of the Lens in Vitro.

The eyes of two chick embryos of about 52 hours incubation (lens vesicle stage) were removed and the anterior third of each eye placed in a homogeneous medium consisting of equal parts of adult chicken plasma and chick embryonic extract. The concave slide method of Carrel was used. The explant was oriented so that the lens vesicle in each could be plainly observed with the microscope. They were allowed to incubate for 72 hours at 37.5°C. and in two of the specimens it was observed that differentiation of the posterior portion of the lens vesicle had occurred, the cells having elongated to form lens cortex. In the other two, due to digestion of the medium, the specimens were not in proper position for observation. Microscopic sections of the specimens were not obtained. Since this work, the author has learned of the explants of chick embryo eyes by Strangeways and Fell (9). The latter workers demonstrated by microscopic sections the differentiation of the eyes which progressed *in vitro* in a surprisingly normal way.

The Cultivation of the Crystalline Lens Epithelium in Vitro.

Experiment I.—The lens and vitreous of chick embryos of from 4 to 9 days incubation at 37.5°C. were removed by incising the posterior portion of the optic vesicle and using traction on the vitreous. Usually a ring of pigmented and nonpigmented epithelium of the rim of the optic vesicle comes away with the lens and vitreous. If the vitreous be excised and the remaining explant cultivated in the manner used by Fischer (7) a growth of epithelium from the iris anlage is obtained.

This experiment demonstrates that the epithelium of the iris anlage can be cultivated *in vitro* and also that if an unmixed strain of lens

epithelium is desired, all extraneous tissue must be removed from the lens. Strains of iris epithelium were carried along in successive subcultures, so that a ready comparison with the cultures of the lens epithelium was always available.

Experiment II.—After many experiments, it was found that the eyes of chick embryos incubated for 5 days at 37.5°C. were ideal for dissecting the crystalline lens free from all extraneous tissue. The eyes usually measured 3 mm. in diameter and the lens slightly less than 1 mm. in equatorial diameter. Both prior to this stage and after it, the protoplasmic adhesions are such as to make it almost impossible to express the lens free from other cells. The embryo is placed in a concave slide and the amnion removed from the eye region. A sharp, cutting needle in a holder is used in each hand, one to steady the eye and the other to incise the posterior portion of the eye and draw out the vitreous on the point of the needle, bringing with it the lens and a portion of the rim of the optic cup. This specimen is placed in Tyrode (10) solution in a clean concave slide, under the binocular dissecting microscope using oculars 2 objectives A2 with transmitted light. The needles are replaced by fresh ones. These are inserted between the equator of the lens and the investing tissue, the other needle gently expresses the lens free from all extraneous tissue. This is transferred to fresh Tyrode solution and then to the embryonic extract-plasma clot medium. After 72 hours incubation it was observed that no outgrowth of cells had occurred.

This experiment demonstrates that it is possible to dissect out the crystalline lens of a chick embryo of 5 days incubation in such a manner as to free it from all extraneous tissue, and that the lens epithelium is contained within a cuticula outside of which when uninjured the cells do not migrate. (Salzmann (11) found that the lens vesicle is soon closed off from the neighboring structures by a cuticula, later the lens capsule.)

Experiment III.—Lenses from 5 day chick embryos isolated in the manner described in Experiment II were incubated *in toto* for 24 hours at 37.5°C. The cultures were then opened and the lenses removed from the media and divided into two pieces. Outgrowths of cells were obtained from the epithelium of two of four lens incubated. One of these, No. 667–2, represented the beginning of a strain of lens epithelium which will be described in Experiment VI as Strain C.

Those incubated for a longer period apparently retained no viable cells as no outgrowths of cells were obtained after section. Some of these specimens took up and retained the neutral red vital stain (1–30,000) during the first period of incubation. The trauma of the expression of these particular lenses may have had something to do with their inability to withstand cultivation outside the

body. Undoubtedly the cuticula of this stage (later the lens capsule) has much to do as a semipermeable membrane with the process of nutrition of the embryonic lens and a slight injury to this membrane would destroy the possibility of its living *in vitro*.

24 hours is sufficient time for incubation of tissue from a 5 day embryo to exclude any growth of cells extraneous to the cuticula of the lens. In our experiments on tissue from this age embryo, growth if it is to occur, will be evident after 24 hours.

This experiment demonstrates that it is possible to rule out tissue extraneous to the cuticula of the embryonic lens by cultivation and then by section of the lens to obtain a culture of an unmixed primary culture of epithelium from the interior of the embryonic lens.

Cultivation of Lens Epithelium in Vitro.

Experiment IV.—Lenses of 5 day embryos were expressed on numerous occasions in the manner described in Experiment II. Having been placed in fresh Tyrode solution, and again replacing the needles for fresh ones, the lenses were incised anteriorly and the lens epithelium stripped off easily to the equatorial region, as there exists a potential space at this stage between the lens epithelium and the cortex. The lens epithelium is then explanted on the point of a needle directly to the embryonic tissue juice-plasma clot medium. The cortex is explanted into another slide. Incubated at 37.5°C.

After 12 to 24 hours the lens epithelium begins to migrate, divide by mitosis, and multiply. After 48 hours observations are recorded. Apparently the entire explant lives, but cells do not wander out from all parts. This depends on the particular conditions of the explant, foldings, the exposure of the cells, the trauma, and the conditions of the media. Many bizarre shapes have been observed. Small pieces of epithelium do as well as large ones. Cells apparently temporarily united move out from the explant, never losing contact, however. The walls of cells in apposition are difficult of definition in the living cultures, but apparently the cells do not form a syncytium. Both membrane and peninsula formation are observed. The explant remains transparent and the newly formed cells are transparent. The wall of the healthy cell is a geometric line. Protoplasmic processes of various lengths are seen, some being very long. The cytoplasm is clear, containing granules some of which take up neutral red in weak solution and others which do not. The mitochondria are visible in the unstained cells. When the microscope substage diaphragm is opened and slight excess light admitted, the cells become invisible. The nucleus is ovoid and usually contains one or two nucleoli. All stages of mitoses may be observed. About the entire living culture there is observed a halo of transparency of the media, indicating either, the influences of the cell products on the media or, the preparation of the media by the

cells for their nutriment. The outgrowth of the cells is apparently preceded by a process of digestion of the media.

Fresh explants of lens epithelium should be cultivated on No. 0 glass cover-slip and examined with the aid of high magnification to prove the absence of any contaminating cells. Fibroblasts can be differentiated and the living pigment epithelial cells of the eye if present will show definite completely opaque rod-shaped ovoid or rounded pigment granules. The nature of these cells from the iris anlage and their appearance when cultivated *in vitro* must be thoroughly studied by anyone attempting to cultivate lens epithelium.

The explants of cortex do not grow. No cells wander or divide. Partial solution of the explant takes place by a process of hydrolysis. Disintegration of the cells occurs and the explant has a fibrillated, washed out appearance. This process can be best observed by allowing the cortex to remain in Tyrode solution and observing it with the microscope. Hydration and swelling will occur quickly, a beaded appearance is produced by globules forming in the cells. These globules rupture, the cell bursts, and the cytoplasm goes out into the solution which is isotonic for other tissues but hypotonic for lens cortex. A solution containing 1.2 per cent NaCl is isotonic for the lens cortex in that this hydrolysis does not take place, but cell migration and division do not occur. The cells are, even at this early stage of differentiation, biologically old. They have a different osmotic coefficient than the anterior lens epithelial cells.

This experiment demonstrates that lens epithelium when cultivated *in vitro* under proper conditions will migrate, divide, and multiply, and that under these same conditions, the lens cortex cells will not do so. A distinction is drawn between lens epithelium which is limited to the anterior and equatorial regions of the lens and the lens cortex which constitutes the differentiated bulk of the lens. They are both epithelial. From the anatomic arrangement of the cells the anterior epithelium and their elongation at the equatorial region passing over into the lens cortex, it may be safe to assume that the cells of the cortex are derived from the epithelial layer, or at least from those cells which are at the equator. It may be possible to grow the cells of the cortex, as long as they retain their nuclei, under different conditions of cultivation. Alterations have been made in the media, but no successful cultures have been obtained. These facts if substantiated by further experiment would lend proof to the idea that the anterior epithelium of the lens differentiates into the cortex.

Experiment V.—Extract of the 9 day chick embryo was used in place of extract of the whole body. It was made by grinding the eyes in a Wedgwood mortar

with pestle and alkali-free ground glass. It was diluted with Tyrode solution and centrifuged. The explants grew well when this was used in place of the whole body extract, but were not free from retinal pigment rods in the cells. Now either pigment cells had been explanted with the lens, or the cells were transferred with the eye extract, or the pigment rods in the eye extract were ingested by the cells. It has already been demonstrated that the lens can be explanted free from pigment rods and these could be seen with the aid of oil immersion lens. It was therefore determined to use extract of the embryo body minus the eyes. This has been done and transparent growths free from any retina pigment rods obtained. Dr. Albert H. Ebeling has been kind enough to examine some of our cultures and has found no contaminating cells in one produced by our perfected technique.

This experiment demonstrates that lens epithelium can be cultivated in media containing extract from the embryonic eye alone and also from the embryonic body minus the eyes. In the latter media, the cells can be proved free from any contaminating cells.

Experiment VI.—To produce an unmixed strain of lens epithelium which can be carried along indefinitely and used for controlled experiments.

Living explants from lenses isolated and cultivated as described in Experiment IV can be divided and two subcultures obtained. These can in turn be subdivided after cultivation and thus secondary and tertiary cultures obtained. By this means a strain of lens epithelium can be propagated from an original single explant. One of our strain (C) was obtained as described in Experiment III after having incubated the entire lens proving the absence of any cells extraneous to the cuticula. The history of this strain may be reported as exemplifying the culture of lens epithelium under these conditions.

June 28, 1926 (No. 667–3). Entire lens explanted from a 5 day embryo.

June 29 (No. 669–2). The lens remains transparent. Dissected out of the media. Cortex shelled out. Found granular, partially disintegrated. Epithelium subcultivated.

July 1. Several processes. Cellular outgrowth from explant.

July 3. Small growth, vacuolated cells. Media changed.

July 6. Good growth of cells. Membrane and peninsula formation.

July 7, 9, 12, 14, 16, 19, 21, 23, 26, 28, 30, Aug. 2, 4, 6. Media changed and culture divided and subcultivated.

One-half of the culture was put on mica cover-slip and one-half on glass cover-slip. Latter examined on the ensuing working day with oil immersion lens and then fixed and stained for further study. The culture on mica was used for the propagation of the strain. Extract of the embryonic body minus eyes was used throughout. The divided cultures about doubled in size every 48 hours. The cells are transparent and remain epithelial in type. They do not dedifferentiate.

This experiment demonstrates that lens epithelium cultivated *in vitro* can be carried on for successive generations. Strain C was carried through seventeen generations over a period of 6 weeks and was thriving when the work was discontinued, August 9. It was then ready for controlled experiments and observations on the rate and character of the growth, the cellular reactions, and the substances produced by the cells.

CONCLUSIONS.

1. Differentiation of the posterior cells of the lens vesicle into lens cortex has been observed *in vitro.*

2. It is possible to dissect out the lens of the 5 day embryo chick in such a manner as to free it from all extraneous cells.

3. Lens epithelium even at a very early stage is contained within a cuticula.

4. Lens epithelium under proper conditions will live, migrate, divide, and multiply *in vitro* in primary explants.

5. An unmixed strain of lens epithelium can be propagated from a primary explant by successive subcultures. This strain can be utilized for controlled experiments on the nutrition of the cells of the lens.

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EXPLANATION OF PLATE 30.

FIG. 1. Fresh explant of lens epithelium. Zenker acetic-Harris hematoxylin. × about 175.

FIG. 2. Showing mitosis and membrane formation in a strain of lens epithelium. × about 355.

FIG. 3. Cells from tip of peninsula in a strain of lens epithelium showing fine protoplasmic processes. × about 495.

FIG. 4. Cells from tip of peninsula in a strain of lens epithelium. Herniations of cytoplasm as first stage of degeneration of these cells. × about 495.

FIG. 1.

FIG. 2.

FIG. 3.
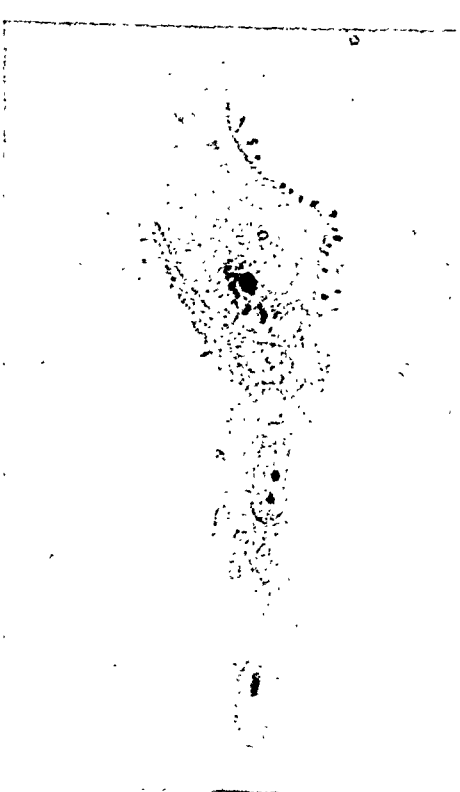
FIG. 4.

(Kirby: Cultivation of lens epithelium.)

THE ACTION OF THE SYMPATHETIC UPON THE EXCITATORY PROCESS IN THE MAMMALIAN HEART.

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The heart *in situ* is normally under the balanced control of its extrinsic nerves, preganglionic fibers of the parasympathetic system which enter *via* the vagus and end about ganglia in the cardiac tissue, and postganglionic fibers of the sympathetic group which are widely distributed to the muscular and specialized tissues.

The influence of the vagus upon the origin and propagation of the excitatory process has been studied in detail. In the mammalian heart it acts to slow the spontaneous rhythm whether this arise in the sinoauricular or auriculoventricular node. The sinus is chiefly under the control of the right nerve while the A-V node is governed by both (1). Vagus stimulation brings about slowing of A-V conduction, the left nerve being the more effective (2). It may also cause partial block in the bundle branches (3). Intraauricular conduction is retarded in the cold blooded heart by vagus stimulation (4). In the auricle of the dog the normal conduction is not altered but if the transmission has been previously slowed it is accelerated by the vagus (5). The refractory period of the auricular muscle is shortened (6) while that of the ventricle is unaffected (7).

While it appears that all portions of the heart are under the influence of the sympathetic its action is most effective at the normal centers of rhythm, the sinoauricular and auriculoventricular nodes (8). At these points there exists a noticeable difference between the influence of the right and left nerves, the right producing primarily an acceleration of the sinus rhythm while the left enhances A-V conduction (9, 10). And finally, when the sympathetic endings are stimulated by means of adrenalin, conduction within the ventricle is accelerated (11).

The present study was undertaken in an attempt to examine certain phases of the action of the sympathetic which have thus far escaped careful investigation.

Effect of Sympathetic Stimulation on Intraauricular Conduction.

The influence of adrenalin upon the transmission of the excitatory process in the auricle after functional exclusion of the vagus, has not to our knowledge been reported. We have, therefore, determined the intraauricular transmission rate before and after adrenalin in a series of eight experiments.

The animals were anesthetized with urethane and the hearts exposed *in situ*. Direct leads were taken from the right auricle by means of non-polarizable electrodes. Adrenalin in 1/50,000 dilution was injected into the femoral vein.

The results of three such observations are shown in Table I. Beginning within ½ to 1 minute after the intravenous injection of adrenalin there is a rise in transmission rate of from 100 to 400 mm. per second, varying with the amount of drug injected but occurring in some cases after doses too small to cause any considerable increase in the rate of the sinus rhythm.

Paralysis of the Sympathetic.

Dale (12) has prepared from ergot a substance, ergotoxin, which consistently depresses the functions of the sympathetic system. More recently Stoll (13) has isolated a pure alkaloid, ergotamine, having similar properties but in greater dilution. This substance antagonizes the pressor action of adrenalin upon the systemic arteries, and inhibits its dilator effect upon the coronaries (14). In dilution 1/100,000 it neutralizes the accelerating action upon the frog's heart of 1/2,000,000 adrenalin (15). It depresses the tendency of adrenalin to produce hyperglycemia in rabbits (16) and relieves the inhibitory action of that drug upon the gut (14). In short, ergotamine has a paralyzing effect upon the sympathetic endings similar to that exerted by atropine upon those of the vagus.

Ergotamine tartrate (0.25–1.0 mg.) was injected intravenously in cats and dogs and the heart rate recorded by galvanometric records.

TABLE I.

Effect of Adrenalin upon Intraauricular Transmission.
Normal Sinus Rhythm.

	Time	Rate	Transmission rate	Remarks
			mm. per sec.	
Experiment 4. Vagi excluded. Electrodes 20 mm. apart	2.35	216	1083	
	2.47	218	1056	
	2.48	210	1065	
	2.49½			0.25 cc. adrenalin 1/50,000
	2.50	220	1170	
	2.51	214	1160	
	2.55	214	1167	
	2.59	210	1121	
	3.02			0.5 cc. adrenalin 1/50,000
	3.02½	216	1153	
	3.03	206	1257	
	3.07	206	1145	
	3.12	206	1029	
	3.14			1.0 cc. adrenalin 1/50,000
	3.15	212	1397	
	3.19	202	1107	
	3.24	206	1100	
Experiment 6. Vagi excluded. Electrodes 20 mm. apart	2.43	179	892	
	2.45	169	865	
	2.47	170	841	
	2.48½			0.5 cc. adrenalin 1/50,000
	2.49	192	1245	
	2.49½	207	1260	
	2.50	200	1038	
	2.51½	178	957	
	2.53	172	915	
	2.58	173	835	
	3.04	170	861	
Experiment 7. Vagi excluded. Electrodes 20 mm. apart	2.58	194	803	
	3.00	185	875	
	3.10	183	723	
	3.12½			1.0 cc. adrenalin 1/50,000
	3.13	191	972	
	3.13½	192	1034	
	3.14	186	1005	
	3.16	182	936	
	3.18	179	897	
	3.23	171	819	
	3.28	169	806	

The animals were anesthetized with urethane and artificial respiration maintained throughout the observation.

In a series of five experiments carried out on animals with the vagi intact the observations of previous workers (14) were confirmed, namely that ergotamine slows the rate of beat and that this slowing is relieved by atropine.

Thus in the experiment outlined in Table II, the injection of 0.25 mg. ergotamine was followed by a slowing of the sinus rhythm from 222 to 136 per minute. A second dose soon after the first failed to

TABLE II.

Effect of Ergotamine upon Sinus Rhythm with Vagi Intact.
Experiment 5.   Cat 1650 Gm.

Time	R-R	Rate	P-R	Remarks
3.48	.26	230	.07	
3.49	.27	222	.07	
3.50				0.25 mg. ergotamine
3.52	.40	150	.08	
3.54	.44	136	.08	
3.55	.40	150	.08	
3.56				0.25 mg. ergotamine
3.58	.44	136	.08	
4.00	.40	150	.09	
4.02				0.25 mg. atropine
4.04	.25	240	.10	
4.06	.25	240	.10	
4.10	.25	240	.10	

produce any pronounced change. After 0.25 mg. atropine, however, the rate rose again to 240.

Such results are obviously susceptible of two explanations: either that ergotamine stimulates the vagus, or that it acts to diminish the accelerating action of the sympathetic. In an attempt to analyze the effect more closely ergotamine was administered after the vagi had been functionally excluded. Both nerves were sectioned in the neck and atropine sulfate, 2.0 mg., was injected intravenously. Following this procedure ergotamine (0.5–1.0 mg.) still produced conspicuous slowing of the sinus rhythm and a prolongation of the P-R interval of the electrocardiogram as shown in Table III. Moreover,

TABLE III.

Effect of Ergotamine upon Sinus Rhythm with Vagi Functionally Excluded.

	Time	Rate	P-R	Remarks
Experiment 9. Dog 5.0 kilos.	2.36	167	.09	
Vagi excluded	2.39	167	.09	
	2.40			1.0 mg. ergotamine
	2.41	125	.12	
	2.42	125	.12	
	2.44	120	.12	
	2.46	118	.12	
	2.51	115	.12	
Experiment 10. Dog 6.2	3.10	260	.07	
kilos. Vagi excluded	3.13	250	.07	
	3.14			0.5 mg. ergotamine
	3.15	200	.08	
	3.16	187	.11	
	3.19	187	.09	
	3.21			1.0 cc. adrenalin 1/10,000
	3.22	187	.08	
	3.23	182	.08	
	3.25	182	.10	
Experiment 12. Dog 7.4	12.10	240	.08	
kilos. Vagi excluded	12.14	250	.08	
	12.15			0.5 mg. ergotamine
	12.16	200	.10	
	12.17	187	.10	
	12.19	172	.11	
	12.25	167	.11	
	12.28			1.0 cc. adrenalin 1/10,000
	12.29	177	.10	
	12.32	167	.12	
Experiment 13. Dog 6.5	2.10	222	.07	
kilos. Vagi excluded	2.12	220	.07	
	2.14	222	.08	
	2.15			1.0 mg. ergotamine
	2.16	194	.10	
	2.17	188	.11	
	2.18	182	.10	
	2.19	182	.10	
	2.21	167	.10	

TABLE IV.

Effect of Ergotamine upon Intraauricular Transmission.
Normal Sinus Rhythm and Rhythmically Driven Heart.

	Time	Rate	Trans-mission	Remarks
			mm. per sec.	
Experiment 26. Vagi excluded. Electrodes 21 mm. apart. Normal sinus rhythm	2.28	230	878	
	2.30	244	910	
	2.31			1.0 mg. ergotamine
	2.33	214	834	
	2.38	209	836	
	2.48	204	788	
	2.53	182	803	
	3.00	172	796	
Experiment 27. Vagi excluded. Electrodes 24 mm. apart. Normal sinus rhythm	2.37	135	922	
	2.40	139	955	
	2.42	136	949	
	2.43			1.0 mg. ergotamine
	2.47	119	918	
	2.51	123	885	
	3.10	135	809	
	3.40	128	847	
	4.10	122	· 817	
	4.40	121	733	
Experiment 19. Vagi excluded. Electrodes 24 mm. apart. Heart rhythmically driven	3.00	153	732	
	3.01	153	762	
	3.03	153	751	
	3.04			1.0 mg. ergotamine
	3.05	153	678	
	3.07	153	674	
	3.10	153	644	
	3.18	153	636	
	3.22	153	646	
Experiment 20. Vagi excluded. Electrodes 26 mm. apart. Heart rhythmically driven	3.23	214	863	
	3.24	214	816	
	3.26	214	877	
	3.27			1.0 mg. ergotamine
	3.28	214	699	
	3.29	214	624	
	3.34	214	635	
	3.38	214	620	·
	3.43	214	623	

TABLE IV—*Concluded.*

	Time	Rate	Trans-mission	Remarks
			mm. per sec.	
Experiment 25. Vagi ex-cluded. Electrodes 24 mm. apart. Heart rhythmically driven	2.47	216	970	1.0 mg. ergotamine
	2.50			
	2.55	216	1040	
	2.58	216	997	
	3.00	216	995	
	3.05	216	847	
	3.12	216	781	
	3.20	216	788	

the subsequent injection of adrenalin in doses of 1.0 cc. of a 1/10,000 solution failed to cause acceleration. Such results indicate that ergo-tamine may effect slowing of the sinus rhythm and cause delay in A-V conduction quite apart from any action upon the vagus. Finally a series of twelve observations was carried out to determine the action of ergotamine upon intraauricular conduction. The results in Table IV indicate that, whether the auricles are responding to the normal sinus rhythm or to rhythmic induction shocks, ergotamine brings about a slowing in the rate of transmission of the excitatory process of from 100 to 200 mm. per second. In the majority of in-stances this effect comes on gradually and reaches its maximum from 20 to 30 minutes after the injection.

DISCUSSION.

When the action of the parasympathetic nerves has been excluded by means of atropine it is possible to demonstrate that the excitatory process in the heart is affected to a considerable degree by the control of the sympathetic nerves. Upon all phases of excitation and con-duction in the mammalian heart with the exception of intraauricular conduction the action of the vagus and sympathetic are opposed. Here the vagus is without effect unless the rate of transmission is abn-ormally slow. Under such circumstances it may bring about accelera-tion but never to a rate above the normal. Stimulation of the sym-pathetic nerves on the other hand invariably causes a conspicuous rise in the transmission rate. It is therefore apparent upon closer analysis

that the effects of the vagus and sympathetic nerves upon intraauricular conduction are in no sense synergistic.

Under normal conditions there exists a delicate balance of such a nature that stimulation of one system involves inhibition of the other. It is, however, significant that, following paralysis of both sympathetic and parasympathetic nerves, the normal rhythm is maintained spontaneously at a more or less constant rate.

SUMMARY AND CONCLUSIONS.

Stimulation of the sympathetic nerve endings, after paralysis of the vagus, accelerates the transmission of the excitatory process in the dog's auricle.

Following the functional exclusion of the vagus ergotamine slows the rate of the sinus rhythm, depresses A-V conduction, and delays the transmission of the excitatory process in the auricle.

The relation of the sympathetic and parasympathetic nerves in the control of the cardiac rhythm is discussed.

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EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS.

THE ELIMINATION OF TUBERCLE BACILLI IN THE FECES, BILE, AND URINE OF INFECTED GUINEA PIGS.

BY DAVID PERLA, M.D.

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The study of an induced epidemic of spontaneous tuberculosis in guinea pigs, the results of which have been reported in a previous paper (1), has shown that the portal of entry of the infection is with few exceptions the intestinal tract, the mesenteric lymph nodes being secondarily involved. It has seemed desirable to determine whether the elimination of tubercle bacilli in the feces of experimentally infected guinea pigs is a source of infection and causes the spread of spontaneous tuberculosis.

It has been demonstrated that bacteria introduced into the circulation may be eliminated through the intestinal tract. Emmerich (2) in 1885 recovered the cholera vibrio from the intestinal tract of cats 6 hours after intravenous or subcutaneous inoculation. Hess (3) in 1910 found that *B. prodigiosus* introduced into the circulation is eliminated from the blood through the liver and the kidneys 1 hour after its injection and even after he tied off the duodenum just below the common bile duct and the canal of Wirsung he was able to recover the bacilli in the small intestine 2 hours after injection. Following the ligation of the common bile duct in guinea pigs, Ribadeau-Dumas and Harvier (4) were able to isolate *B. paratyphosus* from the intestinal tract 24 hours after intravenous injection. Breton, Bruyant and Mézie (5), repeating the experiments of Hess, recovered *B. prodigiosus* from the bile from $\frac{1}{2}$ hour to 24 hours after intravenous injection of 1 mg. of a 24 hour culture. They cite an experiment in which they recovered tubercle bacilli from the bile at the end of 6 hours.

Though the literature[1] on the elimination of tubercle bacilli by tuberculous patients and cattle is extensive, there is no study which

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[1] The literature on the elimination of tubercle bacilli in man and cattle is reviewed by Calmette in "Tubercle bacillus infection and tuberculosis in man and animals," translation by Soper, W. B., and Smith, G. H., Baltimore, 1923, p. 444.

demonstrates that tubercle bacilli are eliminated in the feces of patients or cattle when tuberculous lesions are not in open communication with the surface. When a definite communication exists between a caseous focus and the intestinal tract, it is obvious that tubercle bacilli will be eliminated in the feces.

The Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

An attempt was made to determine the significance of fecal contamination as a source of infection in the spread of spontaneous tuberculosis in guinea pigs.

Experiment 1.—Five guinea pigs were injected intraperitoneally with 0.001 mg. of a glycerol agar transplant 3 weeks old made from a virulent human strain of tubercle bacilli (P 15-B). One normal guinea pig received 0.01 mg. of the same culture subcutaneously. These animals were used in the epidemiological experiments described in a previous paper (1). The feces of these six guinea pigs were tested every week.

If the abdomen of a guinea pig is gently massaged 1 hour after feeding, fecal boluses are readily passed. These boluses, in the absence of an enterocolitis, are firm and uniform in size. Two boluses from each of the guinea pigs tested were collected in sterile Petri dishes. The feces of each animal were ground up in a test-tube with a small quantity of sterile saline solution and the emulsion was filtered through several layers of gauze. Twice the volume of 3 per cent sodium hydroxide was added to the tube and the whole was incubated during 1 hour at 37°C. in a water bath. The emulsion was then centrifuged and the supernatant liquid decanted. The sediment was neutralized against phenolphthalein solution (1 per cent) with 6 per cent hydrochloric acid. The volume was then brought to 5 cc. with sterile saline solution. 2 cc. of this emulsion was injected into the subcutaneous tissue of the thigh of each of two guinea pigs. Monthly intradermal tuberculin tests with 0.02 cc. of "old tuberculin" were made on all the test pigs. All test pigs were killed at the end of 3 months. The results of this experiment are given in Table I. All of the animals of which the feces were tested died of generalized tuberculosis.

Tubercle bacilli have passed through the tissues of the normal animal and are eliminated during the 1st week of infection. The development of immunity against the microorganism may later cause fixation of the bacilli within the tissue thus preventing their elimination. With dissemination of the infection, bacilli are again eliminated.

In the following experiment all animals were inoculated with the

TABLE I.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

	Tubercle bacilli in the feces tested at intervals indicated below:						
	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	
Guinea Pig 1, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	+	X	X	Died at end of 7 wks.
Guinea Pig 2, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	+	X	0	0	0	Died after 13 wks.
Guinea Pig 3, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	+	+	X	Died after 8 wks.
Guinea Pig 4, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	0	0	X	Died after 14 wks.
Guinea Pig 5, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	X	X	X	Died after 4 wks.
Guinea Pig 6, 0.01 mg. *B. tuberculosis*, subcutaneously	X	X	0	+	+	X	Died after 14 wks.

X = no test made on this date. + = tuberculosis present in the test animals.
0 = no evidence of tuberculosis in the test animals.

TABLE II.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs

	Tubercle bacilli in the feces tested at intervals indicated below:							
	Before inoculation	1 day	2 days	4 days	1 wk.	3 wks.	5 wks.	
Guinea Pig 7, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	Died after 51 days of tuberculosis
Guinea Pig 8, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	Killed after 56 days of generalized tuberculosis
Guinea Pig 9, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	+	0	0	0	0	+	Died after 56 days of tuberculosis
Guinea Pig 10, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	+	Died after 52 days of tuberculosis

dose of tubercle bacilli used in Experiment 1 (Guinea Pig 6 excepted).

Experiment 2.—Four guinea pigs received 0.001 mg. of tubercle bacilli (P 15-B) injected into the peritoneal cavity. After intervals of 1, 2, 4, 7, 21 and 35 days the feces of each of these animals were collected and injected into two normal guinea pigs.

The foregoing experiments were not carried beyond the 6th week, and the following experiment was performed in order to collect more information concerning the elimination of tubercle bacilli.

Experiment 3.—Four guinea pigs with negative tuberculin reactions were inoculated with human tubercle bacilli of a glycerol agar growth (P 15-B) 3 weeks old, as follows:
Guinea Pig 11 received 0.001 mg. intraperitoneally and died during the 9th week.
Guinea Pig 12 received 0.00001 mg. intraperitoneally and died at the end of the 11th week.
Guinea Pig 13 received 0.01 mg. subcutaneously in the left thigh, and died at the end of the 7th week.
Guinea Pig 14 received 0.0001 mg. subcutaneously in the left thigh, and died at the end of the 13th week.
All of these animals died of disseminated tuberculosis. On the day when the feces were collected the animals were tested with 0.02 cc. of tuberculin. The procedure was that described in Experiment 1.

None of the guinea pigs inoculated with feces taken during the first 4 weeks of the infection developed tuberculosis. It is however noteworthy that the only animal in Experiment 3 comparable with those of Experiments 1 and 2 is Guinea Pig 11, but in this instance an early elimination of tubercle bacilli in the feces did not occur. Guinea Pig 12 received a very small intraperitoneal injection and in Guinea Pigs 13 and 14, injections were subcutaneous.

Tubercle bacilli were eliminated in the feces of the animals in Experiment 3 during the last few weeks of the infection. The elimination of tubercle bacilli occurred just before and during the interval when the tuberculin reaction was diminishing in intensity or was negative. The number of experiments, however, is not sufficient to establish definitely the relation of the tuberculin reaction to the terminal elimination of tubercle bacilli. The negative tuberculin reaction has been a manifestation of overwhelming infection and only after the disease is apparently well advanced and disseminated, are tubercle

bacilli eliminated in the feces in sufficient quantities to infect guinea pigs.

The Elimination of Tubercle Bacilli in the Bile of Experimentally Infected Guinea Pigs.

There have been numerous studies of the elimination of tubercle bacilli in the bile.

Calmette and Guérin (6) found that tubercle bacilli are eliminated in the bile of tuberculous rabbits. They injected a series of rabbits intravenously with 10 mg. of bovine tubercle bacilli and killed them at intervals of 24 and 48 hours, 3, 4, 5, 6 and 7 days. In each instance, the contents of the gall bladder were aspirated and injected into guinea pigs. Tubercle bacilli were recovered from the bile obtained on the 3rd and 7th days. By establishing a permanent biliary fistula in a heifer (7), they were able to make daily tests of the bile aspirated through the fistulous opening. Following intravenous injection of 3 mg. of virulent bovine tubercle bacilli, they found that tubercle bacilli were eliminated in the bile only after 19 days. The heifer died of miliary tuberculosis 26 days after inoculation. In a series of twenty-four guinea pigs with disseminated tuberculosis killed at a time when death was expected as a result of the infection, Remlinger (7) tested the contents of the gall bladder for tubercle bacilli by direct inoculation into guinea pigs and observed eighteen positive results. Sasano and Medlar (8) found that the appearance of tubercle bacilli in the bile of experimentally infected guinea pigs is indicative of tuberculosis of the liver and is usually evidence of an ulcerative tuberculous lesion of the biliary system. From the time the bile duct becomes involved until death, there is a continuous elimination of tubercle bacilli into the intestinal tract (9). The exact time of appearance of the tubercle bacilli in the bile is not stated in their work.

Tubercle bacilli have been found in the bile of patients dying of pulmonary tuberculosis by Fraenkel and Krause (10) and by Corper, Simmons and Freed (11). In patients suffering with tuberculosis tubercle bacilli have been discovered in the bile aspirated through a duodenal tube, by Carnot and Libert (12), Freed and Black (13), Olmer and Crémieux (14) and others. There is, however, no evidence recorded in the literature which conclusively demonstrates the elimination of tubercle bacilli through the biliary system in the absence of lesions of the liver or of the biliary tract.

To explain the spread of spontaneous tuberculosis it is desirable to determine when tubercle bacilli appear in the bile of infected guinea pigs and to compare the elimination in the bile with that in the feces. It may be that this is the main source of tubercle bacilli when the disease is disseminated and involves the liver. The attempt has been

TABLE III.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

Tubercle bacilli in the feces tested at intervals indicated below:

	24 hrs. before inoculation	24 hrs. after inoculation	2 days	4 days	6 days	11 days	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.	9 wks.	10 wks.	11 wks.	12 wks.	13 wks.
Guinea Pig 11 0.001 mg. *B. tuberculosis,* intraperitoneally	0	0	0	0	0	0	0	0	0	0	+	0	+					
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	3+	2+	4+	4+	3+	1+					
Guinea Pig 12 0 00001 mg. *B. tuberculosis,* intraperitoneally	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+		
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	2+	4+	4+	3+	4+	3+	1+	Neg.		
Guinea Pig 13 0.01 mg. *B. tuberculosis,* subcutaneously	0	0	0	0	0	0	0	0	0	0	+	+						
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	2+	2+	3+	4+	2+						
Guinea Pig 14 0.0001 mg. *B. tuberculosis,* subcutaneously	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	1+	3+	4+	3+	3+	2+	2+	Neg.	Neg.	Neg.

The intensity of the tuberculin reaction is indicated by 1+, 2+, 3+ or 4+.

made to study the elimination of tubercle bacilli in the bile of infected guinea pigs throughout the course of the infection.

Experiment 4.—Nine normal guinea pigs of the same weight and age and from the same stock were inoculated subcutaneously with 0.001 mg. of the strain of virulent human tubercle bacilli (P 15-B) used in the previous experiments. These animals were killed at intervals of 24 and 48 hours, 4, 7, 14, 21, 28 and 42 days. The last animal died after 44 days. The animals were chloroformed and the ab-

TABLE IV.

	Interval between infection and death in days	Tubercle bacilli in bile	Tubercle bacilli in urine	Remarks
Guinea Pig 15 not infected		0	0	No tuberculosis
Guinea Pig 16	1	0	0	No tuberculosis
Guinea Pig 17	2	0	0	No tuberculosis
Guinea Pig 18	4	0	0	No tuberculosis
Guinea Pig 19	7	0	0	No tuberculosis
Guinea Pig 20	14	+	0	Tuberculous lesion at site of injection and in regional lymph nodes
Guinea Pig 21	21	+	0	Tuberculous lesion at site of injection and in inguinal, iliac and lumbar lymph nodes. Spleen enlarged but liver apparently normal
Guinea Pig 22	28	+	0	Same as Guinea Pig 17
Guinea Pig 23	42	+	0	Tuberculosis of spleen, liver and lungs
Guinea Pig 24	44	+	+	Tuberculosis of lymph nodes, liver, spleen and lungs

domen opened with aseptic conditions. The gall bladder was brought prominently into view, the surface thoroughly seared and the entire contents of the bladder aspirated. The organs of these animals were carefully examined and sections were made from the liver, kidney and site of inoculation.

The total volume was brought up to 4 cc. with sterile saline solution and injected subcutaneously into two guinea pigs. These test guinea pigs were killed as soon as the tuberculin reaction was strongly positive or at the end of 3 months. The results of this experiment are given in Table IV.

Tubercle bacilli were eliminated in the bile of the experimentally infected guinea pigs from the 14th day until death.

The following experiment was made to determine whether tubercle

bacilli were eliminated with the bile during the first few hours following inoculation.

Experiment 5.—Guinea pigs were injected into the peritoneal cavity with 0.01 mg. of a culture of *B. tuberculosis*, and after intervals of 4, 8, 12, 24 and 48 hours the bile of each of these pigs was collected and injected into two normal pigs. The results of this experiment are given in Table V.

After subcutaneous inoculation of tubercle bacilli with the dose used (0.01 mg.) tubercle bacilli have appeared in the bile only once within the first 2 days after injection.

TABLE V.

	Interval between infection and death in hrs.	Tubercle bacilli in bile
Guinea Pig 25	4	0
Guinea Pig 26	4	0
Guinea Pig 27	8	0
Guinea Pig 28	8	0
Guinea Pig 29	12	0
Guinea Pig 30	12	0
Guinea Pig 31	24	+
Guinea Pig 32	24	0
Guinea Pig 33	48	0
Guinea Pig 34	48	0

Elimination of Tubercle Bacilli in the Urine of Experimentally Infected Guinea Pigs.

Remlinger (7) in a series of twenty-four guinea pigs with disseminated tuberculosis killed at a time when death was expected found that the urine aspirated from the urinary bladder in half of the animals, contained virulent tubercle bacilli, demonstrated by animal inoculation.

Although there are numerous clinical studies demonstrating the presence of tubercle bacilli in the urine of individuals in whom there has been no evidence of renal tuberculosis, it is not improbable that transient bacteremia has caused minute focal lesions of renal glomeruli which have healed or have been overlooked at autopsy.

An attempt has been made to determine whether tubercle bacilli eliminated with the urine of tuberculous guinea pigs experimentally

infected for the elimination of tubercle bacilli in the urine, may facilitate the spread of spontaneous tuberculosis. In guinea pigs used in Experiment 4, the urinary bladder was seared and the urine was aspirated aseptically. The total volume from each animal was brought up to 4 cc. with sterile saline solution and injected subcutaneously into two guinea pigs. The results of this experiment are given in Table IV.

The urine aspirated from the bladder of infected guinea pigs did not contain tubercle bacilli until the terminal stage of the infection. Guinea Pig 24 died 44 days after inoculation and the urine aspirated from its bladder caused tuberculosis in guinea pigs.

DISCUSSION.

In the course of experimental infections in animals and in spontaneous disease in man, tubercle bacilli doubtless appear intermittently in the blood stream.

Calmette (15) in a review of the literature of bacteremia with tuberculosis maintains that with experimental tuberculous infection of animals bacteremia it present before a lesion appears at the site of inoculation and continues throughous the infection. A large quantity of blood may be necessary to show presence of tubercle bacilli. Calmette demonstrated tubercle bacilli in the blood stream by direct transfusion of 10 cc. of blood from a tuberculous into a normal guinea pig. This huge quantity of blood is approximately equivalent to a liter of human blood.

Bergeron (16) states that bacteremia in experimental tuberculosis of rabbits occurs soon after injection and is ephemeral.[2] Haga (18) tested the blood of guinea pigs infected subcutaneously with virulent human tubercle bacilli. He found by animal inoculation and by staining methods that tubercle bacilli were present in the blood stream during the first 10 days after inoculation in five of twelve experiments. The number of positive results diminished from the 10th to the 30th day, and almost wholly disappeared during the interval from the 30th to the 40th day. From the 40th day until death bacilli were again discovered in the blood stream in increasing numbers and were present in two-thirds of the animals examined. In 66 per cent of instances, they appeared between the 50th and the 70th day after inoculation.

In normal rabbits, Opie (19) has demonstrated that antigens such as horse serum or egg white are demonstrable in the blood during a period from approximately 7 to 9 days after injection. "With the progress of immunization injected antigen

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[2] The literature of the subject is thoroughly reviewed by Löwenstein (17) and by Calmette (15).

exhibits a decreasing tendency to find its way into the circulating blood so that in a well immunized animal horse serum or egg white injected into the subcutaneous tissue is not demonstrable in the blood serum even if tests are made at intervals from 1 to 24 hours after injection." In a subsequent study it has been found that the antigen introduced into an immune animal is fixed at the site of injection (20).

In the studies of Krause (21) and Willis (22) on the spread of tubercle bacilli in normal and immune guinea pigs infected by subcutaneous inoculation, it has been demonstrated that tubercle bacilli inoculated intracutaneously or subcutaneously into normal guinea pigs are carried to the regional lymph node within an hour, and within 3 or 4 days they have been disseminated throughout the body and may be isolated from the lungs, hilum lymph nodes and other tissues. In immune animals their spread is greatly retarded. They remain fixed at or near the site of entry during about 7 days, do not reach the regional lymph nodes until 2 weeks after infection and are not disseminated throughout the body until 2 or 3 weeks after infection. "The immune state (that is, the allergic reaction) operates to retard the spread of the bacilli . . . . rather than to inhibit . . . . their activities or their multiplication" (21).

The observations described in this paper show that tubercle bacilli may be eliminated with the feces during the 1st week following inoculation. At a later period elimination ceases and does not begin again until the disease is far advanced. Elimination by way of the bile occasionally occurs immediately after. inoculation, but is insignificant until tuberculosis is well established in the body. Elimination by way of the urine has occurred only when the disease has reached its terminal stage.

Tubercle bacilli appear in the blood stream of infected guinea pigs during the first 10 days following subcutaneous injection, but gradually disappear and reappear after about 40 days (Haga). Elimination of microorganisms in the bile of rabbits injected intravenously with large quantities of tubercle bacilli occurs within 3 days (Calmette and Guérin), and as early as 6 hours (Breton, Bruyant and Mézie). The evidence presented by these investigators and the observations reported in this paper show that tubercle bacilli introduced forcibly into the tissues of normal animals enter the blood stream and are eliminated in the feces before immunity against the microorganism is established. With the progress of immunization the bacilli become fixed in the tissues, are no longer capable of permeating intact mucous membranes and do not appear in the feces. When the infection is widely disseminated, resistance is overcome and the elimination of tubercle bacilli recurs.

CONCLUSIONS.

1. After intraperitoneal inoculation of guinea pigs with tubercle bacilli, the microorganisms may appear in the feces and elimination may persist during 1 week. Subsequently during several weeks no tubercle bacilli can be recovered from the feces.

2. When tuberculosis is widely disseminated in the body of guinea pigs, tubercle bacilli are discharged with the feces.

3. Tubercle bacilli are occasionally eliminated in the bile of guinea pigs immediately after inoculation but are almost constantly found in the bile after several weeks when the disease is well established.

4. Tubercle bacilli are eliminated in the urine of experimentally infected guinea pigs only when infection is far advanced.

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BACTERIOPHAGE ISOLATED FROM THE COMMON HOUSE FLY (MUSCA DOMESTICA).

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INTRODUCTION.

It is known, through the work of others, that the house fly, when fed cultures of various pathogenic bacteria, harbors them for a longer or shorter time but that they finally disappear entirely. Various explanations of this elimination have been offered or are suggested by the facts presented. Wollman (1) regarded the process as entirely mechanical. In the case presented by Jones and Little (2) an actual rapid sterilization was observed in which more subtle factors are obviously concerned.

Glaser and Sanderson, in unpublished observations from this laboratory, found in house flies bacteriophage active against *Staphylococcus muscæ*, a microorganism which gives rise to a definite disease of this insect (Glaser (3)). This observation offered the suggestion that bacteriophage might be found more widely active and might be concerned in the natural process of removal of foreign bacteria when these are fed to the fly. Duncan (4) has recently studied the same problem and discovered a bactericidal principle active against many species of bacteria in the gastrointestinal tracts of various insects, the fly among them. The references above generally commented on are more specifically as follows.

Graham-Smith (5) found that, with flies fed on cultures, *B. prodigiosus* (page 96) could survive in the crop only about 17 days. *B. enteritidis* Gärtner (page 146) could be recovered from the gut contents up to the 7th day. *B. typhosus* (page 130) up to the 6th day, and *V. choleræ* (page 173) for 2 days after ingestion by the insect. Manson-Bahr (6) found that *B. dysenteriæ* Shiga, from a culture, survived in the gut contents of flies 4 days. Naturally infected flies, he thought, carried the organ-

isms for a much longer time. Wollman (1) observed that flies fed on cultures of *B. typhosus* and *B. dysenteriæ* and then transferred daily to aseptic surroundings became free of the specific bacteria in from 8 to 10 days. Jones and Little (2), in conducting an investigation on infectious ophthalmia of cattle, made the observation that the causative diplobacillus was not capable of surviving as long as 5 minutes in the gastrointestinal tract of the house fly. Duncan (4) isolated a bactericidal principle from the alimentary tracts of a number of insects and arachnids. He included *Musca domestica* in the series of insects found by him to contain this bactericidal principle in their gastrointestinal contents. He considered that this principle might be a bacteriophage but was forced to discard the possibility as the substance he described exhibited none of the essential properties of this principle.

The experimental work recorded in the following pages was conducted with the purpose of determining any bactericidal action which might pertain to the house fly, and its result has been to identify bacteriophage very active against a number of bacteria, as well as an inhibitory substance, not identified with bacteriophage, and active still more widely.

EXPERIMENTAL.

1000 house flies were obtained from the vicinity of a hog lot, etherized and ground in a mortar with physiological salt solution in the proportion of 10 flies per cc. This mixture was filtered first through paper, then through a Berkefeld N filter. A clear, dark, straw-colored fluid was obtained which gave no bacterial growth when incubated on the ordinary culture media. This fluid was kept at refrigerator temperature and was used as the starting point for most of the experimental work here reported.

Two organisms were used at the outset to determine whether or not the fly filtrate just described contained substances which were either bactericidal or capable of inhibiting bacterial growth. One of these was a non-mucoid strain of *B. coli* 223 of calf origin (7), and the other *B. paratyphi* Type I of guinea pig origin (8).

Plain bouillon (pH 7.5), 5 cc. per tube, was used as the culture medium and the fly filtrate was added to the bouillon before inoculation. 1, 0.5, 0.25, and 0.1 cc. amounts of fly filtrate were added to four separate tubes in each series and one tube in each was kept as a control. One loopful of a 24 hour bouillon culture was used in making each inoculation. The tubes were read at 6 and 24 hours with the results given in Table I.

This experiment made it evident that the fly filtrate contained some substance which was inhibitory or bactericidal for *B. coli* (calf) but

which exerted no such action upon *B. paratyphi* Type I (guinea pig).
This apparent specificity suggested the possibility that the factor
might be bacteriophage. Each tube of the two sets of cultures was
therefore passed through a Berkefeld N filter and the experiments were

TABLE I.

	B. paratyphi Type I (guinea pig)		*B. coli* (calf)	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
cc.				
1	Moderately turbid	Turbid	Clear	Clear
0.5	" "	"	"	"
0.25	" "	"	"	"
0.1	" "	"	"	"
Control	" "	"	Moderately turbid	Turbid

TABLE II.

Dilution	*B. paratyphi* Type I (guinea pig)			*B. coli* (calf)		
	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.
10^{-1}	C.*	M.T.	M.T.	C.	V.S.T.	S.T.; agglutinated
10^{-2}	"	"	"	"	"	" "
10^{-3}	"	"	"	"	"	" "
10^{-4}	"	"	"	"	S.T.; agglutinated	M.T.; "
10^{-5}	S.T.	S.T.	S.T.	"	" "	" "
10^{-6}	"	"	"	"	" "	" "
10^{-7}	M.T.	"	"	"	" "	" "
10^{-8}	"	"	"	S.T.	" "	" "
10^{-9}	"	"	"	"	" "	" "
Control	"	T.	T.	M.T.	T.	T.

* C. = clear; V.S.T., very slightly turbid; S.T., slightly turbid; M.T., moderately turbid; T., turbid.

repeated using the bouillon filtrate. The dilutions were made this
time from tube to tube increasing by successive powers of ten. A single
pipette was used for each series. The results are given in Table II.

After 48 hours incubation, each tube of these two sets of cultures
was passed through a Berkefeld N filter and the experiment repeated
with results similar to the above.

TABLE III.

Dilution	B. typhosus (Rawlings)			B. paratyphi Type I (guinea pig)			B. coli (calf)			Staphylococcus musca		
	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.
10^{-1}	C.*	C.; Sed.	C.; Sed.	C.	M.T.	M.T.	C.	C.	S.T.	C.	C.	C.
10^{-2}	"	" "	" "	S.T.	"	"	"	S.T.	"	"	"	"
10^{-3}	"	" "	" "	"	"	S.T.	"	"	"	"	"	"
10^{-4}	"	" "	" "	T.	"	"	"	"	"	"	"	"
10^{-5}	"	" "	" "	"	T.	M.T.	"	"	"	"	"	"
10^{-6}	"	" "	" "	"	"	"	"	"	"	"	"	"
10^{-7}	"	" "	" "	"	"	"	"	"	"	S.T.	"	"
10^{-8}	"	S.T.; "	S.T.; "	"	"	T.	S.T.	"	"	"	"	"
10^{-9}	S.T.	T.	T.	"	"	"	M.T.	M.T.	"	"	"	"
10^{-10}	"	"	"	"	"	"	"	T.	T.	"	M.T.	M.T.
10^{-11}	M.T.	"	"	"	"	"	"	"	"	"	T.	T.
10^{-12}	"	"	"	"	"	"	"	"	"	"	"	"
Control	"	"	"	"	"	"	"	"	"	"	"	"

* C. = clear; S.T., slightly turbid; M.T., moderately turbid; T., turbid; Sed., sedimentary growth.

Plates were next made and these showed typical plaque formation in both series. Individual colonies on solid media showed notched and stellate forms. The inhibition of growth in series by dilutions of the filtrates and the formation of plaques definitely characterizes the action as that of bacteriophage.

The activity of the original fly filtrate against a wider range of bacteria was then tested. Preliminary determinations were carried out using a single pipette for each set of dilutions, while the experiments to determine the definite end-points of activity, those given in Tables III and IV, were done using a fresh pipette for each dilution.

TABLE IV.

Organism	Highest dilution showing inhibition of growth at 6 hrs.
Staphylococcus aureus	1:1000 (complete)
Streptococcus C 54	1:100 "
" 744	1:10 (complete). 1:10,000 (partial)
" C 55	1:10,000 (partial)
Pneumococcus I	1:10 (complete). 1:10,000 (partial)
" II	1:10,000 (complete)
Bacillus of swine plague	1:1000 (partial)
Vibrio choleræ	No inhibition of growth
Friedländer's bacillus	" " " "
Bacillus proteus	.. " " " "

Whenever evidence of inhibition of growth was obtained filtrates were made and tested in order to develop any potential transmission in series. Inhibition, displayed in successive filtrates, was thus uncovered in the case of B. typhosus (Rawlings) and Staphylococcus muscæ in addition to the two species previously considered. The complete record, as finally determined for the four species, is shown in Table III.

The original filtrate inhibited the growth of certain other species in various dilutions but filtrates in these cases had no inhibitory action. The end-point of activity for these bacteria and the species for which no inhibitory action could be demonstrated are contained in Table IV.

The bacteriophage isolated was very active against Staphylococcus muscæ, giving complete inhibition of growth to a dilution of 10^{-9}. The lysis of this organism was complete and permanent and no secondary growth ever occurred in the tubes that were completely lysed at 48

hours. It was fairly active against *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-8}, but in the case of this organism all lysed tubes showed some secondary growth in the form of a sediment. Against *B. coli* (calf) it was also fairly active, giving evidence of lysis in a dilution of 10^{-9}. With this organism a secondary growth occurred in 24 hours also. The lytic principle active against *B. paratyphi* Type I (guinea pig) was a relatively weak one and a secondary growth always occurred. Lysis in this case seemed to progress for at least 48 hours but complete clearing of the cultures never resulted.

The nature of the inhibitory action which differs so definitely from bacteriophage is of considerable interest. One possibility seemed to be that it might be related as a precursor or "building stone" for the bacteriophage. To test this, one of the species susceptible to inhibition, but for which bacteriophage was not developed, was fed to flies for a period. These flies were extracted as before and the filtered extract was tested for the characteristic transmissible lysis for the bacterium fed. The result was negative. The detailed experiment follows.

Streptococcus C 55, a non-hemolytic strain originating in bovine mastitis (9), which was inhibited but not susceptible to lysis by the bacteriophage in the original fly filtrate, was used. About 200 flies were placed in a large glass jar and fed bouillon cultures of Streptococcus C 55 daily for 8 days. At the end of this time the flies were etherized and ground in a mortar with physiological salt solution using 5 flies per cc. This material was passed through a Berkefeld N filter for sterilization and, from the filtrate, attempts were made to obtain bacteriophage capable of lysing cultures of Streptococcus C 55. These attempts were all unsuccessful and no bacteriophage active against this organism could be obtained.

DISCUSSION.

Physiological salt solution extracts of the house fly present bacteriolytic or inhibitory phenomena of two types which may have a bearing on the inability of certain pathogenic microorganisms to exist for more than a short period of time in the gastrointestinal tract of the insect. Bacteriophage active against at least four species of bacteria was found in a salt solution extract of flies; and another substance, growth-inhibiting, but not showing the essential characteristics of bacteriophage, was also present. This was active against four additional species.

In so far as relates to the bacteriophage, it is very likely that the fly filtrate contains a mixture of lytic principles, rather than a single bacteriophage capable of causing lysis of the four species. Thus neither the broth filtrate active against *B. coli* (calf) nor the one active against *B. paratyphi* Type I (guinea pig) had any lytic action on *Staphylococcus muscæ*. The filtrate active against *B. paratyphi* Type I (guinea pig) was also strongly lytic for *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) was somewhat active for *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-4}. The filtrate active against *Staphylococcus muscæ* failed completely to cause lysis of *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) caused no lysis of *B. paratyphi* Type I (guinea pig). No effort was made to adapt a lytic principle, active against one organism, to another of the group.

Because of the possibility that the non-bacteriophagic growth-inhibiting substance might be a precursor to true bacteriophage, the feeding experiment described was planned. No bacteriophage against the streptococcus fed could be obtained in this way. It is very likely that this non-bacteriophagic growth-inhibiting factor is the same as that observed by Duncan (4).

Within the range of the experimental observations, the lytic principle present in the fly filtrate may have been obtained either from the external parts of the fly or from its digestive tract. A much more elaborate technical procedure would be required to make this discrimination.

SUMMARY AND CONCLUSIONS.

1. Bacteriophage active against four species of bacteria was found in a salt solution extract of house flies.

2. A growth-inhibiting principle, not bacteriophage, active against four other species of bacteria was found to be present in the same extract.

3. An attempt to secure streptococcus bacteriophage by feeding to flies a streptococcus susceptible to the inhibitor but not to the bacteriophage of this filtrate was unsuccessful, indicating that the two activities are quite unrelated.

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ON THE INFLUENCE OF ACID GROUPS ON THE SEROLOGICAL SPECIFICITY OF AZOPROTEINS.*

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PLATE 31.

In previous work (2–5) investigations were made of the relation between chemical constitution and serological specificity by compounding various substances of simple chemical structure with proteins and using the resulting combinations as antigens. The coupling of proteins with diazonium derivatives of aromatic substances offered itself as the most suitable means. The immune sera obtained were tested by the precipitin reaction. In order to avoid species-specific reactions on the protein component the test antigens generally were prepared with a protein from another species than that employed for the immunizing antigens. The principal result of these studies was the observation that the immune sera exhibit a specificity which is determined by the chemical structure of the simple compounds attached to the protein.[1]

Aside from the phases already dwelt upon there are others to which the method outlined is applicable. The present paper deals with the influence of acid groups on specificity. Reference has been made to this question in one of the papers quoted (3).[2] For our purpose antigens were prepared with chemicals with and without acid groups; after immunization with these substances the immune sera were tested with both types of antigens.[3]

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* Twenty-second paper on antigens and specificity. *Cf.* Reference 1.

[1] For a fuller review we refer to "The chemical aspects of immunity," by H. Gideon Wells, American Chemical Society Monograph Series, New York, 1925, page 77.

[2] Page 387.

[3] For the sake of brevity these will be referred to in the following as acid and non-acid antigens and immune sera respectively.

EXPERIMENTAL.

Preparation of the Antigens for Immunization.—As an example the method used for aniline is given. The other substances were taken in quantities equivalent to that of the aniline.

1.2 gm. of aniline were dissolved in 10 cc. of water and 5 cc. of 7 N HCl and diazotized with the necessary amount of sodium nitrite at a temperature of 0–5°C., with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 100 cc. and poured into a mixture of 100 cc. of horse serum and 100 cc. of normal sodium carbonate. The solution must give a strongly alkaline reaction with phenolphthalein. Coupling was allowed to take place for 10 minutes at 0–5°C. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was suspended in a small amount of water. On addition of some normal sodium hydroxide and vigorous stirring it became viscous or jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the material.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol it was brought into solution with alkali as before and after dilution it was reprecipitated with hydrochloric acid and filtered. After grinding in a mortar the substance was dissolved in water at alkaline reaction. Finally the reaction was adjusted with hydrochloric acid to faint alkalinity or neutrality.

The volume was made up to 190 cc. with distilled water and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 10 cc. of a 5 per cent phenol solution were added. The presence of some suspended azoprotein in the solution does not affect its use for the immunization.

The other preparations were made in a similar way. Some of them such as the azoproteins from ortho- and para-chloroaniline are still less soluble, therefore a great part of the substance remains in suspension. The diazotization of para-nitroaniline was carried out by adding at room temperature the sodium nitrite solution to a suspension of the finely powdered substance containing the required amount of hydrochloric acid. Small amounts of undissolved material were removed by filtration. This same method was applied to ortho-nitroaniline and 1, 2, 5-nitrotoluidine which were only used for the preparation of test antigens. In the case of the amino acids[4] as para-arsanilic acid, para-aminobenzoic acid, etc., the azoproteins can be dissolved without difficulty. For coupling diazotized ortho-aminobenzoic acid with horse serum, 50 cc. of normal NaOH per 100 cc. serum were used instead of sodium carbonate.

Immunization.—Eight rabbits were injected intraperitoneally with 15 cc. or less of each antigen at weekly intervals. At least two immune sera of sufficient strength were obtained after three to six injections in every case. Only one serum was obtained in the instance of para-chloroaniline.

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[4] For further details see Reference 3, pages 354–364.

Antigens for the Tests.—The azoproteins for the test solutions were prepared with chicken serum in place of horse serum in the manner described above. The diazo solution (in a few instances containing undissolved diazo compound) was added to a mixture of serum and sodium hydroxide (1 part of normal sodium hydroxide to 2 parts of serum) and the coupling allowed to take place at room temperature for half an hour. After precipitation with dilute hydrochloric acid the azoprotein was filtered and washed with water.

There was some difficulty in that the preparations did not yield clear solutions or were only sparingly soluble. This was overcome by treatment of the azoprotein with alkali. An amount of the antigen corresponding to 5 cc. serum was ground in a mortar, made up to 10 cc. with water and 10 cc. of normal NaOH was added. After half an hour the mixture was neutralized with 10 cc. normal HCl, the precipitated azoprotein centrifuged and taken up in about 25 cc. saline containing 0.5 cc. normal NaOH. After a few minutes hydrochloric acid was added until the solution was only weakly alkaline to litmus paper and the volume made up to 30 cc. The solution to which 0.5 per cent phenol had been added was clarified by intense centrifugalization and filtration through kieselguhr paper.[5]

To make the tests comparable also the antigens with acid azo components were treated with alkali in the same manner (concerning their preparation see Reference 3, pages 362 and 363). To obtain solutions of antigens made from the halogen substituted anilines it was necessary to prolong the treatment with alkali. A quantity of the azoprotein corresponding to 5 cc. serum was ground and 20 cc. of normal NaOH added. The mixture was shaken with beads for 2 hours, neutralized and the precipitated azoprotein was taken up in 25 cc. of saline containing 0.5 cc. normal NaOH. After addition of hydrochloric acid until the solution was weakly alkaline to litmus it was brought up to 30 cc., centrifuged and filtered through kieselguhr paper.

The quantity of antigen present in the solutions was determined by precipitating the azoprotein with alcohol, drying and weighing.

The solutions were found to contain from about 20 to 45 mg. of dry material in 5 cc.

Tests.—The antigens were diluted to 1:100 of a 1 per cent solution and 0.2 cc. of this solution was used for the tests. The tests were kept at room temperature and were in some instances also read after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), ±, +, + ±, etc.

Tests with Azoproteins Made from Aniline and Various Substituted Anilines.

Tables I and II illustrate the action of various immune sera on the azoproteins prepared from aniline and a number of substituted anilines.

[5] Macherey, Nagel and Co., Düren, Germany.

TABLE I.

Of the immune sera 779 and 788 four drops were used, and of the others 2 drops. The readings were made after 1 hour at room temperature.[6]

Azoproteins prepared from chicken serum and:	Immune sera obtained from antigens made from horse serum and:									
	Aniline	Aniline	Ortho-chloroaniline	Ortho-chloroaniline	Para-toluidine	Para-toluidine	Para-toluidine	Para-nitroaniline	Para-nitroaniline	Para-chloroaniline
	No. 760	No. 761	No. 788	No. 793	No. 763	No. 766	No. 770	No. 775	No. 795	No. 779
Aniline.................	++±	+±	+±	++	+±	+±	++	+	±	+
o-Toluidine..............	++	±	++	++±	+±	+	+	±	f.tr.	±
o-Anisidine..............	+	0	+±	++	±	tr.	0	0	0	0
o-Nitroaniline...........	+	0	+	+	tr.	tr.	0	±	tr.	0
o-Chloroaniline..........	+±	±	++	++±	+	+	+	±	+	tr.
m-Toluidine..............	++	+	+±	++	+±	+	+±	+	+	+
m-Nitroaniline...........	+	tr.	+	+	+	±	+	+±	+±	±
m-Chloroaniline..........	++	+	+	+±	+±	+	+±	+	+	+
m-Bromoaniline..........	++	+	+	++	+±	+	+	+	+	+
p-Toluidine..............	+±	+	+	+	++	+±	++±	+	+	++
p-Anisidine..............	++	±	±	+	++±	+±	++±	+	+±	+±
p-Nitroaniline...........	+	±	±	tr.	+±	+	+±	++	++	++
p-Chloroaniline..........	++±	+	±	+	++	+±	++±	+±	++	++
p-Bromoaniline..........	++	+	+	+±	++	++	++±	+±	++	++
p-Iodoaniline............	+±	+	+	+	++	+±	++±	+±	+±	++

TABLE II.

1,2,4-Nitrotoluidine	++	±	+	++	+±	+±	++	+±	+±	+
1,4,2-Nitrotoluidine.....	++	±	+±	+∓	++	+	+±	+±	+±	+
1,2,5-Nitrotoluidine.....	+	tr.	±	tr.	+	tr.	±	+±	+±	±
1,3,4-Xylidine	+±	±	+±	++	++	+±	++	+	+	+±
1,4,5-Xylidine..........	+±	tr.	+±	++	+	+	+	+	+	tr.
1,4,2-Xylidine..........	+±	tr.	+±	++	+	+	+	+	±	tr.
Acetyl-para-phenylene-diamine...............	0	0	0	0	0	0	0	0	0	0
Para-aminoacetophenone.	tr.	0	0	0	+	tr.	±	+	±	0
Monomethyl-para-pheny-lenediamine...........	+±	+				+±	++	+±		

[6] The sera and the antigens are indicated by the names of the substances used in their preparation.

The experiments summarized in Table I show that the immune sera precipitate nearly all antigens. Also in most cases of negative reactions distinct precipitation occurred after standing overnight in the ice box and the weak reactions increased considerably in strength. Nevertheless a certain degree of specificity is to be observed.

The homologous reaction is always one of the strongest. The nature of the substituent is generally of but little influence; in the tests with

TABLE III.

4 drops were used of immune serum 729 and 2 drops of the others. The readings were made after 1½ hours at room temperature.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Azoproteins made from chicken serum and:										
		Para-aminobenzoic acid	Meta-aminobenzoic acid	Ortho-aminobenzoic acid	Para-arsanilic acid	Sulfanilic acid	Ortho-cinnamic acid	Aniline	Para-nitroaniline	Ortho-nitroaniline	Para-toluidine	Meta-toluidine
Para-amino-benzoic acid..	816	++±	±	0	0	0	0	0	0	0	0	0
Para-amino-benzoic acid..	818	+±	.0	0	0	0	0	0	0	0	0	f.tr.
Ortho-amino-benzoic acid..	729	0	0	++	0	0	0	±	±	±		±
Para-arsanilic acid.........	722	0	0	0	++++	0	0	0	0	0		f.tr.
Aniline	760	0	0	0	0	0	0	++±	+	+.	+±	++
Para-nitro-aniline.......	775	0	0	0	0	0	0	+	+±	+±	+	+
Para-toluidine..	770	0	0	0	0	0	0	++	+±	0	++±	+±

several immune sera however the intensity of the reactions was more diminished by the presence in the azo component of a nitro or a methoxy group than by the other substituents tested.

More pronounced is the effect produced by the position of the substituents regardless of their nature. In the tests with immune sera against para substituted azoproteins the strength of the reactions decreases in a general way in the order para, meta, ortho. This sequence is reversed in the reactions with sera against the ortho sub-

stituted aniline. Observations similar to these have been made previously (3, 5). The aniline immune sera seem to react somewhat more weakly with ortho substituted antigens than with the others.

No definite regularities appear from the tests of Table II except for the negative or weak reactions with the antigens prepared from acetyl-para-phenylenediamine and para-aminoacetophenone. The other antigens behave similarly to those included in Table I.

Tests with Azoproteins Prepared from Amino Acids.

A considerable number of immune sera prepared with acid azoproteins were described previously (3).

TABLE IV.

2 drops of aniline immune serum No. 760 were used. Readings after 1 hour.

Azoproteins made from chicken serum and the following substances:	Antigen dilutions 1 to			
	100	300	1000	3000
Aniline..............................	+++	++±	+	±
Ortho-nitroaniline.....................	+	+	tr.	0
Meta-nitroaniline.....................	++	+±	±	tr.
Para-nitroaniline.....................	+±	+±	±	tr.
Ortho-anisidine......................	+	+	±	tr.
Para-anisidine.......................	+±	+±	±	f.tr.
Para-aminobenzoic acid...............	tr.	0	0	0
Sulfanilic acid.......................	tr.	0	0	0

The present experiments as recorded in Table III demonstrate the considerable influence of acid groups on the specificity.

Table IV gives the results of tests made with various concentrations of the antigens.

The sera prepared with "non-acid" antigens give no precipitation with the "acid" azoproteins.[7] In the converse experiment showing the action of the "acid" immune sera on the "non-acid" antigens, there were weak positive reactions especially with one of the four sera and

[7] Distinct precipitation occurs in higher concentration of the acid antigens. These reactions were shown to be non-specific flocculations since such reactions took place also with entirely unrelated immune sera as precipitins for rat, pig serum, etc.

some more weak reactions came up after keeping the tests overnight. The reactions with serum No. 729 increased somewhat in strength.

A representative experiment is illustrated by Figs. 1 and 2.

In conformity with the findings already referred to (3) the sera for acid antigens exhibit in general a considerably higher specificity than the others. This is further substantiated by a series of tests in which the antigens for the reaction *in vitro* were made with the same protein as that used for the immunization (horse serum). Also in this way the difference between the two sorts of antigens is brought out clearly (Table V; *cf.* Fig. 3) although the method would tend to destroy the specificity of the reactions, due to the fact that the immune sera react to a certain degree upon the protein part of the antigens.

TABLE V.

To 0.2 cc. of the diluted antigen 1 drop of immune serum was added. The first reading was taken after 5, the second after 15 minutes.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Antigens made from horse serum and:										Horse serum	
		Aniline		Para-toluidine		Para-nitroaniline		Para-arsanilic acid		Para-amino-benzoic acid			
Aniline.....	760	++±	+++	+±	++	++	+++	tr.	±	0	tr.	±	+
Para-toluidine..	763	++	++	++	+++±	++	++	tr.	tr.	0	0	+	+
Para-nitro-aniline....	735	+±	+++±	+	++	++	+++	tr.	±	0	±	tr.	±

Reactions with an Azoprotein Made from the Methyl Ester of Para-Aminobenzoic Acid.

In order to prove in a different way the results obtained the following experiments were carried out. Starting from the ester of an aromatic amino acid an azoprotein was prepared. Presumably this should react with the non-acid immune sera like the other non-acid antigens. If this azoprotein is treated in such a way as to bring about hydrolysis of the ester it should no longer react with such sera but should have acquired by virtue of the free carboxyl group the property of reacting with an immune serum specific for the corresponding acid.

The methyl ester of para-aminobenzoic acid was prepared according to the directions given by Einhorn (6). The recrystallized substance melted at 112°C.

Diazotization.—0.190 gm. of the finely powdered substance was suspended in 10 cc. of water. Normal hydrochloric acid and a 2 per cent solution of sodium nitrite were added alternately in small quantities keeping the solution distinctly acid to Congo red and adding the nitrite after a test with potassium starch iodide paper showed the disappearance of nitrous acid. The theoretical quantity of nitrite was used up. The solution was kept at 0–5°C. This diazo solution was added to a mixture of 10 cc. of chicken serum and about 7 cc. of normal sodium carbonate, cooled to 0–5°C. The mixture was distinctly alkaline to phenolphthalein but an excess of alkali was avoided to prevent hydrolysis of the ester. After coupling for 10 minutes at 0–5°C., and acidifying with hydrochloric the azoprotein was filtered and washed with water. It was made up with saline into a fine suspension (volume 20 cc.) and made slightly alkaline to litmus by addition of 0.1 cc. of normal sodium hydroxide. Undissolved material was removed by centrifuging. The azoprotein was precipitated by neutralization with hydrochloric acid and after centrifugalization it was redissolved in 10 cc. of saline with alkali, carefully avoiding an excess. The solution which was very faintly alkaline to litmus was centrifuged and filtered through kieselguhr paper. Its content of azoprotein was estimated in the manner described before. The test solution was made up to 1/100th of a 1 per cent solution.

The experiment turned out as was anticipated (Table VI). The solution of the ester-azoprotein was precipitated by aniline and para-toluidine immune sera and not by an immune serum for para-aminobenzoic acid except for a weak reaction observed when the tests were kept overnight in the ice box. This is in agreement with the behavior of other non-acid azoproteins.

Hydrolysis of the Ester-Azoprotein.—To 2 cc. of a 1 per cent solution of ester-azoprotein were added 2 cc. of 1/10 normal sodium hydroxide and samples of 0.5 cc. were taken from the mixture at various times, *i.e.*, immediately after mixing, and after being kept 5 minutes, 1, 4, 6, 8 and 20 hours, at room temperature. These samples were adjusted to faint alkalinity with 1/10 normal hydrochloric acid, the azoprotein thus being kept in solution.

Table VII gives the reactions with the two immune sera at various stages of the hydrolysis. The antigen concentration corresponded in all cases to 1/100 of a 1 per cent solution.

TABLE VI.

2 drops of immune serum were used. Readings after 1 hour and after standing overnight in the ice box.

Azoproteins made from chicken serum and:	Immune sera obtained from antigens made from horse serum and:							
	Aniline		Para-toluidine		Para-nitroaniline		Para-aminobenzoic acid	
	No. 760		No. 770		No. 775		No. 816	
	1 hr.	Night	1 hr.	Night	1 hr.	Night	1 hr.	Night
Aniline.................	++	+++±	++	+++±	+	++	0	tr.
Para-toluidine...........	+±	++	++	+++±	+	++	0	tr.
Para-nitroaniline.........	+±	++	+±	++	+±	+++	0	±
Para-aminobenzoic acid...	0	0	0	0	0	0	++	++++
Para-aminobenzoic ester...	+	++	+	++	±	++	0	±

TABLE VII.

2 drops of immune serum were used. Readings after 1 hour at room temperature and after standing overnight in the ice box.

Azoproteins prepared with chicken serum:	Immune sera obtained with antigens prepared from horse serum and:			
	Para-aminobenzoic acid		Para-toluidine	
	No. 816		No. 770	
	1 hr.	Night	1 hr.	Night
Para-aminobenzoic ester azoprotein.....	0	tr.	+±	++
Same immediately after mixing with alkali.............................	f.tr.	±	+±	++
Same after 5 min. hydrolysis..........	±	+	+±	++
Same after 1 hr. hydrolysis.............	+±	+±	+	+±
Same after 4 hrs. hydrolysis.....	+±	++	tr.	+
Same after 6 hrs. hydrolysis...........	++	+++±	0	+
Same after 8 hrs. hydrolysis...........	++	+++	0	tr.
Same after 20 hrs. hydrolysis...........	++	++++±	0	tr.
Para-aminobenzoic acid azoprotein.....	++	++++±	0	tr.
Para-toluidine azoprotein..............	0	tr.	++	+++±
Aniline azoprotein.....................	0	f.tr.	++	+++±

The experiment shows that during the course of the hydrolysis the precipitation with the serum for para-toluidine decreases gradually while correspondingly an increase in the strength of the reactions takes

place with the immune sera for para-aminobenzoic acid. The former reaction did not disappear completely but a trace of precipitate was still noticed after 20 hours hydrolysis when the tests were read on the following day.

<center>SUMMARY.</center>

The method of partial synthesis of antigens as employed in the foregoing experiments obviously cannot be substituted for the chemical study of natural antigens. But some questions of a rather general nature not easily accessible to investigations of the latter sort, may be approached by the use of artificial protein compounds. Thus the results reported indicate a peculiarity of certain chemical structures such as acid radicals.

The group of immune sera obtained by injecting azoproteins made from non-acid azo components had a wide range of activity. Substituents like CH_3, OCH_3, NO_2, Cl, Br, I, in the aromatic nucleus altered the reactions to a moderate degree only, in most cases.[8] The effects were dependent more on the position than on the nature of the substituents. Two substances were found, however, which had a pronounced effect on the specificity of the compound protein, namely acetyl-para-phenylenediamine and para-aminoacetophenone. In consideration of the above facts it is uncertain whether the antigenic changes noticed by Obermayer and Pick (7) after treating proteins with nitric acid, nitrous acid or iodine are mainly due to the substitution of hydrogen in the benzene ring by NO_2 and I, as is the general belief, or to other changes of the protein. This question had been raised already by the observation that the proteins treated with HNO_3 or HNO_2 containing respectively the nitro or the diazo group, did not differ substantially in their serological properties (8).

The antigens made from acid compounds form a group with distinctive features. In the first place the presence of acid radicals destroys the reactivity with the immune sera for the non-acid substances. This influence is so marked that even the reaction with the species-specific part of the protein, if such is present, appears to be diminished. Also the sera produced with the acid antigens react but feebly with the non-acid azoproteins. Accordingly it was pos-

[8] *Cf.* Wells,[1] page 79.

sible to show that by hydrolysis of the ester of an aromatic acid contained in an azoprotein the serological reactions of the antigen underwent a radical change.

The presence of a free carboxyl group in the antigens not only determines the characteristics mentioned but there is reason to believe that it increases markedly the degree of specificity exhibited by the antigens and the corresponding immune sera, when cross-tests are made with a number of acid azoproteins and their antisera. This is brought out by a comparison of the results of the present investigation with those described previously (3). It is of interest in this respect that the specific carbohydrates found by Avery and Heidelberger in pneumococci and pneumobacilli are mostly, if not in all cases, compounds of distinctly acid character.

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EXPLANATION OF PLATE 31.

FIG. 1. Reactions of immune serum for para-toluidine No. 770 with various azoproteins prepared from chicken serum and the following substances, readings after 1 hour:

1. Aniline.
2. Para-toluidine.
3. Ortho-chloroaniline.
4. Ortho-nitroaniline.
5. Meta-nitroaniline.
6. Para-nitroaniline.
7. 1, 3, 4-Xylidine.
8. 1, 2, 4-Nitrotoluidine.
9. Para-arsanilic acid.
10. Para-aminobenzoic acid.
11. Sulfanilic acid.
12. Saline control.

FIG. 2. Reactions of immune serum for para-aminobenzoic acid No. 816 with various azoproteins prepared from chicken serum and the following substances, readings after 1½ hours:

1. Aniline.
2. Para-toluidine.
3. Ortho-nitroaniline.
4. Para-nitroaniline.

5. Ortho-anisidine.	9. Para-aminobenzoic acid.
6. Para-arsanilic acid.	10. Sulfanilic acid.
7. Ortho-aminobenzoic acid.	11. Ortho-aminocinnamic acid.
8. Meta-aminobenzoic acid.	12. Saline control.

FIG. 3. Reactions of immune serum for aniline No. 760 with various azoproteins and with unchanged horse serum, reading after 15 minutes.

1. Azoprotein from chicken serum and aniline.
2. Azoprotein from horse serum and aniline.
3. Azoprotein from horse serum and para-toluidine.
4. Azoprotein from horse serum and para-nitroaniline.
5. Azoprotein from horse serum and para-aminobenzoic acid.
6. Azoprotein from horse serum and para-arsanilic acid.
7. Unchanged horse serum.
8. Saline control.
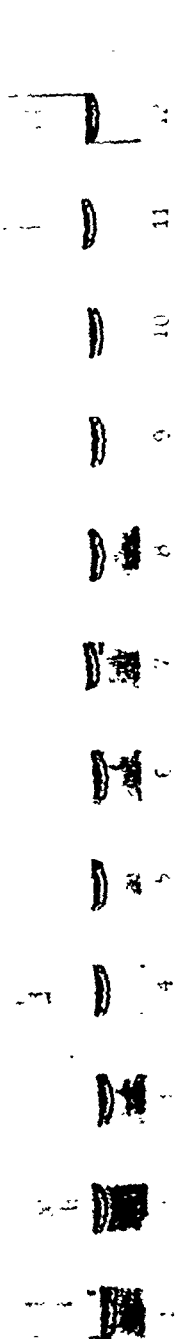
FIG. 1.
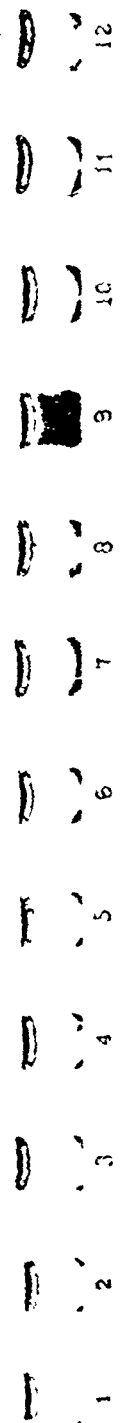
FIG. 2.
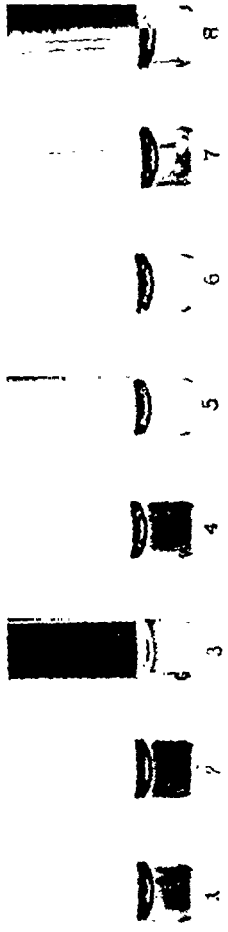
FIG. 3.

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THE DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS FOLLOWING INHALATION OF PNEUMOCOCCI.

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In a preceding paper (1) it has been shown that mice may acquire a high degree of active immunity following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the immunity developed during a series of exposures. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

It has already been shown that rabbits are susceptible to infection by inhalation of Type I pneumococci (2), and that an occasional rabbit may recover from pneumococcus septicemia. In the present paper are reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of virulent Type I pneumococci. The duration of active immunity and the length of time that agglutinins and protective antibodies persist in the serum will be dealt with in a subsequent paper.

Method.

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of virulent Type I pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal.

The presence of agglutinins was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of Pneumococcus Type I. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intra-

which form the basis of this paper, were obtained from rabbits which had not only the greatest natural resistance, but also a certain degree of immunity acquired as the result of repeated exposures to live pneumococci.

In Table I are shown the number of rabbits which were sprayed from 1 to 10 times, the number which died of pneumococcus septicemia, and the number whose serum showed agglutinins in dilutions of at least 1:10. From Table I it is seen that only 4 or 2 per cent of the 135 rabbits which survived the first exposure developed demonstrable agglutinins. The percentage of rabbits showing agglutinins rose after the 2nd spray to 12 per cent, increasing to 25 per cent on and after the 5th spray. In fact 17 rabbits, after having been sprayed 10 times, failed to develop demonstrable agglutinins.

In all, 21 rabbits developed agglutinins. In Table II are shown the exposure after which agglutinins first appeared and the titre of agglutinins following each subsequent spraying. Although 4 rabbits showed agglutinins after the first exposure, a second exposure was necessary to incite the formation of these antibodies in 9 others. 1 rabbit first showed agglutinins only after 7 exposures. Great variations are also seen in the antibody titre of the different animals. As a rule agglutinins were first demonstrable only in the higher concentrations of serum (1:10 or 1:20) but in one instance the reaction first appeared following the 5th spray and was then present in a dilution of 1:100. Following the initial appearance of agglutinins there is a tendency for the titre to rise after the next spray and then to remain stationary. Of 8 rabbits which were sprayed 8 times, only 3 showed an agglutinin titre of 1:100; the serum of 3 others showed a titre as high as 1:50, while in one exceptional instance the serum suddenly showed agglutinins after the 5th spray in a dilution of 1:100 increasing after the 6th spray to 1:200.

Protective Antibodies.

In order to determine whether the serum of normal rabbits contains any natural antibodies against Type I pneumococcus, 1 cc. of serum from each of 147 normal rabbits was injected intraperitoneally into as many mice. The next day these mice were injected intraperitoneally with 0.000,001 cc. of virulent culture of Type I pneumococcus.

136 or 92 per cent of these mice died. In order to determine whether the 11 surviving mice recovered by virtue of a natural antibody present in certain normal rabbit sera, or by reason of a non-specific reaction induced by foreign protein, plain broth and normal horse serum were tested. 25 mice received in like manner injections of 1 cc. of plain broth, and another 25 were injected with 1 cc. of normal horse serum. 24 hours later all 50 mice were inoculated intraperitoneally with 0.000,001 cc. of virulent Type I pneumococcus. Of the mice receiving a preliminary injection of broth 24 or 96 per cent died, and of those injected with horse serum 17 or 68 per cent succumbed to subsequent infection. From the results of this experiment it would seem that the mere prelim'nary injection of a foreign serum of either the horse or rabbit, in certain instances conferred protection in mice against an

TABLE III.

Number of Rabbits Exposed and Number Whose Serum Protected Mice against 0.001 Cc. of Pneumococcus Type I.

No. of exposures.................	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " whose serum protected......................	5	10	14	25	22	22	21	23	19	19
Per cent of rabbits whose serum protected.....................	3	9	16	39	45	61	61	74	76	82

otherwise fatal inoculation of Type I pneumococcus. From this it is evident that the 8 per cent of normal rabbit sera which afforded protection did so not because of the presence of natural antibodies but because of the non-specific protective reaction induced by the foreign serum.

In Table III are shown the number of rabbits which were sprayed from 1 to 10 times with Type I pneumococci and the number thus exposed whose serum subsequently protected mice against intraperitoneal injection of 0.0001 cc. of a virulent culture of the homologous organism. From this table it is seen that protective antibodies were demonstrable in the sera of 5 or 3 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits showing protection steadily increased, until after the 10th spraying the

serum of 82 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. A total of 49 rabbits developed protective antibodies. The spray following which the rabbit sera first protected mice is shown in Table IV.

From Table IV it is seen that with each successive spray the number of rabbits in whose sera protective antibodies were demonstrable progressively increased. Although the greatest number of rabbits showed protective antibodies in their sera after the 6th spray, other rabbits did not develop these antibodies until after the 10th exposure.

Correlation of Agglutinins and Protective Antibodies.

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentrations of 1:10,

TABLE IV.

First Appearance of Protective Antibodies.

No. of spray	1	2	3	4	5	6	7	8	9	10
" " rabbits first showing protective antibodies	5	7	7	12	7	5	1	4	—	2

protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.0001 cc. of virulent pneumococcus culture. If smaller doses of Pneumococcus had been used, the presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of a high degree of immunity. In 10 instances both agglutinins and protective antibodies occurred after the same spray; in 9 animals agglutinins appeared before protective antibodies were demonstrable, while in 30 instances protective antibodies occurred without demonstrable agglutinins in the serum. In the 9 rabbits in which agglutinins appeared first, protective antibodies were later demonstrated in 4 instances after the next spraying, in 2 others after 3 additional sprayings, and in 1 rabbit not until after 5 subsequent exposures.

The relative occurrence of agglutinins and of protective antibodies in the serum of rabbits following inhalations of pneumococci is graphically shown in Text-fig. 1. In this figure the mortality curve of rabbits dying from septicemia is also given. It is seen that after the 1st exposure 34 per cent of rabbits died from pneumococcus septicemia. However, no rabbits died from this cause after the 4th spraying. In other words, the more highly susceptible animals were rapidly elimi-
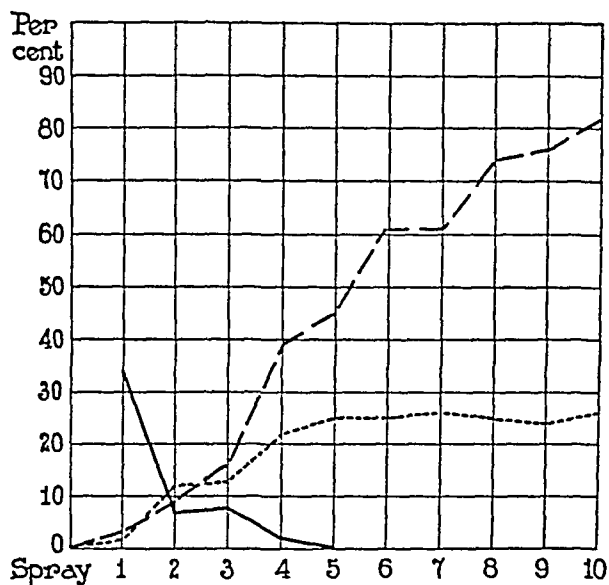
TEXT-FIG. 1. Comparison of mortality, and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type I pneumococci.

———— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

------- per cent of rabbits showing agglutinins in serum following spraying.

— — — per cent of rabbits showing protective antibodies in their serum.

nated by the 1st exposure, while those that survived were either naturally more resistant or had gained some degree of immunity. It is interesting to note that up to the 5th spray an increasing proportion of rabbits developed agglutinins but that after this the percentage remained stationary. On the other hand, with each successive spray an increasing number of rabbits developed protective antibodies until

following the 10th exposure 82 per cent of the total number showed the presence of these immune substances in their serum.

DISCUSSION.

From the foregoing experiments it appears that following repeated inhalations of living Type I pneumococci, rabbits develop a high degree of immunity as evidenced by the presence of demonstrable agglutinins and protective antibodies in their serum. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may even recover from a transient pneumococcus septicemia. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which after implantation are able to invade the tissues, and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS.

1. Following repeated inhalations of Type I pneumococci agglutinins and protective antibodies can be demonstrated in the serum of rabbits.

2. The percentage of rabbits whose serum shows agglutinins remains stationary after the 5th exposure, but the percentage of rabbits showing protective antibodies in their sera steadily rises.

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THE RESPONSE TO RESPIRATORY RESISTANCE.

A COMPARISON OF THE EFFECTS PRODUCED BY PARTIAL OBSTRUCTION IN THE INSPIRATORY AND EXPIRATORY PHASES OF RESPIRATION.

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The liability to fatigue of the respiratory center is a subject which needs to be studied. Davies, Haldane, and Priestley (1) were the first to investigate the manner in which breathing adapts itself to increased resistance, and the point at which the adaptation begins to fail. They showed that the normal response in man to respiratory resistance in both phases of respiration is slow and deep breathing. When the resistance is excessive respirations become progressively shallower and more frequent and the subjects then become cyanotic. Haldane and his coworkers believe that this is due to fatigue of the respiratory center. They believe anoxemia hastens greatly the onset of fatigue and the ease with which it is produced. They conclude that the mechanism involved in the immediate response is the Hering-Breuer reflex, pointing out that as a result of resistance, the time required for inflation or deflation of the lungs to reach the point at which the Hering-Breuer stimulus becomes effective is prolonged, that CO_2 accumulates in the meantime, and that the next respiration is deep and vigorous. The more or less sudden onset of rapid, shallow breathing Haldane interprets as evidence of fatigue of the respiratory center, with a resulting predominance of the peripheral stimuli over the central impulses normally governing breathing.

A study of these effects in animals was undertaken by us with several points in mind. We hoped for additional information as to the nature and origin of rapid and shallow breathing, which we have previously considered in both clinical and experimental studies (2–5). It seemed highly desirable to learn something about the liability to fatigue of so vital a structure as the respiratory center. Transferring the problem

to experimental animals rather than man, though it introduced such complications as the use of anesthetics, afforded the opportunity of allowing the experiments to go to their natural conclusion. It made it possible, too, to study the end-results of more or less prolonged periods of anoxemia and rapid and shallow breathing. It was soon learned that the response to resistance in inspiration is strikingly different from the response to resistance in expiration, both as regards functional and structural changes.

Method.

Dogs anesthetized with barbital-sodium were used. The dogs varied in weight from 6.8 to 23.5 kilos, but in every instance except one the weight of the animal was above 10 kilos. The barbital-sodium was dissolved in physiological sodium chloride solution and given intravenously in an amount sufficient to produce complete relaxation and a slow, steady respiratory rate. The initial dose was calculated on a basis of 0.3 gm. drug to 1 kilo body weight and the additional drug was given in repeated small quantities as necessary.[1] The rectal temperature was recorded at frequent intervals throughout the course of each experiment. As a precaution against the loss of heat each animal was wrapped snugly in woolen blankets and surrounded by warm air [2]

When the animal had reached the desired state of anesthesia, tracheotomy was done, and a properly fitting rubber tube was tied firmly into the trachea. This tube, which was of such a length as not to increase the dog's natural dead space, communicated with one arm of a four-way metal tube. Two of the other branches of the four-way tube were connected by corrugated tubing of the usual type used in respiration experiments to inspiratory and expiratory valves. Low resistance valves of the kind recently described by Dr. C. V. Bailey[1] (6) were used in all experiments.

The fourth opening in the metal tube was connected to a rubber tambour by means of a short length of rubber pressure tubing. This tambour, moving with expiration and inspiration, activated a make and break contact in an electrical circuit which included an electromagnet "telephone" counter. By this arrangement the respiratory rate was automatically counted. Intratracheal pressure was measured by a water manometer communicating by means of a Y-tube with the

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[1] A 5 per cent solution was used for this purpose.

[2] A cradle made by covering an arch of thin metal with hair felt was placed over the animal. Heat was supplied by an electric bulb suspended from the top of the arch. We have found that this is a much more efficacious way of maintaining the warmth of an animal than by use of an electric pad.

[3] These valves were kindly supplied us by Dr. Bailey.

four-way tracheal tube. The expired air was collected in a large Tissot spirometer. To introduce resistances we used a specially constructed metal tap with an
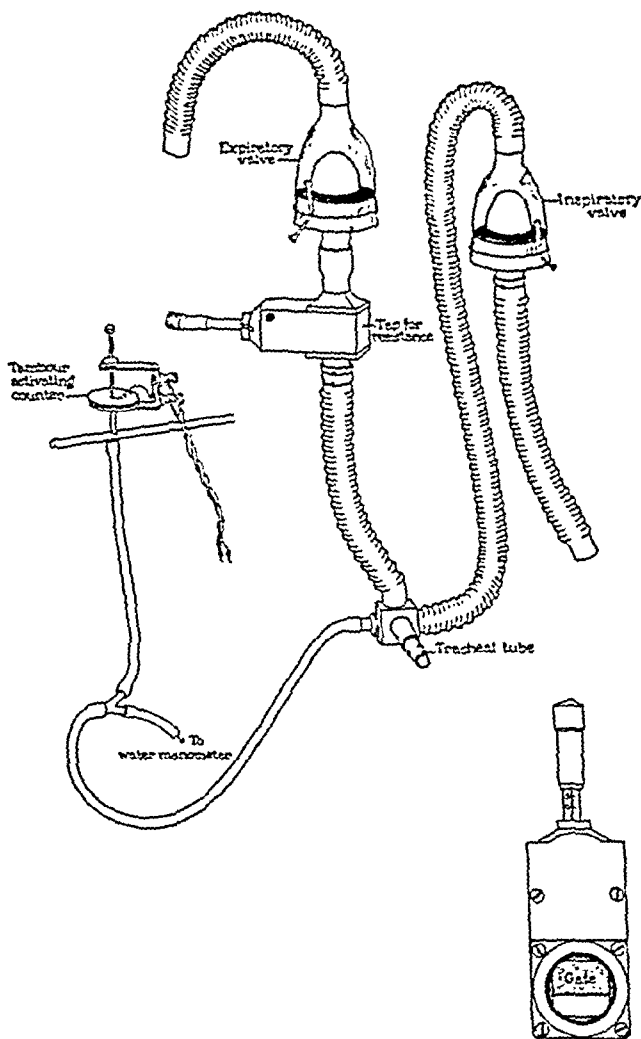
FIG. 1.

internal bore of 4 sq. cm. The tap was closed by means of a gate which was gradually lowered into its seat by a screw of such a pitch that one turn lowered the gate 1 mm. and accordingly diminished the lumen by 0.2 sq. cm. A scale permitted one

to read off at any time the cross-section area in sq. cm. This tap was introduced into the system at a point between the trachea and one of the respiratory valves. According to its position, resistance could be created either in the inspiratory or expiratory phases of respiration. The apparatus which we used is shown in diagram in Fig. 1.

When resistance to respiration is introduced by a tap of this sort the velocity of air flow varies as the square root of the driving pressure. With cotton wool resistance, however, which Haldane and his coworkers used, the air flow varies directly as the driving pressure. The intratracheal pressure may be assumed to represent the driving pressure. Velocity of air flow can thus be calculated if desired. We have chosen tap resistance because it is easier of manipulation and quantitative control. It was found that great reduction in the cross-section area of the tap was necessary before any change in type of breathing occurred.

All experiments were conducted on the same general plan and the observations fall into three periods; first, the period of control; second, the period of resistance; and, third, the period following release.

The observations include a record of respiratory rate, tidal air, minute volume of pulmonary ventilation, intratracheal pressure, cross-section area of the tap, and in some instances an additional study of the O_2 content, O_2 capacity, percentage oxygen saturation, CO_2 content, CO_2 tension, and pH of the arterial blood. The blood samples were withdrawn through a cannula in the femoral artery and collected without exposure to air in sampling tubes over mercury. The analyses of oxygen content and capacity, and of CO_2 content of the separated plasma or serum were made by the method of Van Slyke and Neill (7). The pH was estimated on serum by the colorimetric method of Hastings and Sendroy (8). From these data and the following formula (9) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pK' to be 6.115.

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{\,pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter.

The records of respiratory rate and pulmonary ventilation were made over 5 minute periods, and were repeated several times under each condition. During the time of resistance the cross-section area of the tap was reduced to a degree that created a negative or positive intratracheal pressure of from 10 to 20 cm. of H_2O, and this was maintained over intervals ranging from 20 to 145 minutes. The readings in the final period were taken when the respiratory rate had reached a constant, or approximately constant, level.

In some experiments the animals breathed room air. In others they breathed 90 to 95 per cent oxygen from a Douglas bag. After the final observations the experiment was terminated by the intravenous injection of from 20 to 30 cc. of a saturated solution of magnesium sulfate. At autopsy attention was given to the presence or absence of froth in the trachea, pleural effusions and gross edema of

the mediastinal tissues and lungs, and to the color of the lungs. The degree of hypostatic congestion, the lung weights, the heart weights, and the lung-heart ratio were also observed.

TABLE I.

Experiment 7. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mм*	*mm. Hg*		*mм*	*mм*	*per cent*
Control period	11.57 to 12.46	Room air	19	273	5.11	25.8	32.91	7.50	8.47	9.02	93.9
During resistance	12.46 to 2.55	Room air	30	92	2.78	28.1	53.15	7.32	6.29	9.39	67.0
After release	2.55 to 3.48	Room air	40	225	9.00	24.5	28.63	7.54	8.89	9.66	92.0

Weight of animal, 23.5 kilos.
Total barbital-sodium, 0.35 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 20 cm. H_2O.
Duration of greatest resistance, 113 min.
Lung-heart ratio, 1.66.

EXPERIMENTS.

I. *Experiments with Resistance in the Inspiratory Phase.*

Five experiments were performed in which the cross-section of the tap opening was 0.05 sq. cm. In these experiments the negative intratracheal pressure during the inspiratory phase varied from 11 to 20 cm. of H_2O. In a sixth experiment a conspicuous effect on breathing was obtained with a cross-section area of 0.1 sq. cm. All animals showed an increase in respiratory rate and a decrease in tidal air. The percentage increase in respiratory rate ranged from a low extreme of 58 per cent to a high extreme of 310 per cent, and the percentage

postasis was limited to the dependent parts of the lobes. The lung-heart ratio was 1.66.

In Experiment 1A (Table II) the animal breathed 90 to 95 per cent oxygen. In spite of this, after inspiring against resistance for 21 minutes the percentage oxygen saturation of the arterial blood dropped from 107.8 to 50.4. The rate accelerated from 15 to 47 and the tidal air dropped from 189 cc. to 21 cc. The tension of CO_2 rose from 41.8 mm. to 80.5 mm. and the pH decreased from 7.40 to 7.19. The final estimations in this animal were made 34 minutes after the resistance was removed. It should be noted that the respiratory rate at this time was 139, the tidal air only 75 cc., the tension of carbon dioxide still above the original level, and the percentage oxygen saturation of the arterial blood only 54. Autopsy showed a considerable amount of frothy liquid in the trachea and 10 to 15 cc. of clear fluid in each pleural cavity. The lungs were boggy with fluid.

In Experiment 5 (Table III) the increase in CO_2 tension during the period of resistance was slight, and the pH did not change. The animal breathed air, and the percentage oxygen saturation of the arterial blood dropped from 90.6 to 57.3. This accompanied an increase in respiratory rate from 8 to 17 and a decrease in tidal air from 249 cc. to 80 cc. Resistance was continued 124 minutes. 41 minutes after release all functions were at the normal level. Autopsy in this case showed no froth in the trachea, no effusion into either pleural cavity, and no edema of the mediastinal tissues. The lungs were diffusely coral-pink in color. Hypostatic congestion was slight. The lung-heart ratio was 1.7.

The data for Experiments 2, 3, and 4 are grouped together in Table IV. The results in general are consistent with those obtained in Experiments 1A and 7. The increase in minute volume during the period of resistance in Experiment 3 was a single occurrence. In Experiments 2 and 3 the animals breathed 90 to 95 per cent oxygen and maintained a pink color of the tongue and mucous membranes throughout. It seems safe to conclude that resistance in these animals did not produce anoxemia. At autopsy pleural effusions were found in Experiments 3 and 4. In Experiment 3 there was in addition an excessive edema of the mediastinal tissues. The color of the lungs in all cases was a coral-pink, of varying shades of intensity, but deeper in every instance

TABLE IV.

Experiments 2, 3, and 4.

The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Experiment 2					Experiment 3					Experiment 4				
	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume
				cc.	*liters*				*cc.*	*liters*				*cc.*	*liters*
Control period	2.27 to 2.41	90–95 per cent O_2	18	114	2.09	11.01 to 11.22	90–95 per cent O_2	10	184	1.84	12.03 to 1.29	Room air	19	203	3.86
During resistance	2.41 to 5.15	90–95 per cent O_2	35	50	1.76	11.22 to 2.18	90–95 per cent O_2	41	64	2.62	1.29 to 4.30	Room air	39	80	3.21
After release	5.15 to 6.04	90–95 per cent O_2	44	90	3.98	2.18 to 3.40	90–95 per cent O_2	118	105	12.42	4.30 to 5.14	Room air	34	172	5.84

Weight of animal, 6.8 kilos. Total barbital-sodium, 0.16 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.05 sq. cm. Negative intratracheal pressure, 14.5 cm. H_2O. Duration of greatest resistance, 38 min. Lung-heart ratio, 1.75.

Weight of animal, 17 kilos. Total barbital-sodium, 0.30 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.05 sq. cm. Negative intratracheal pressure, 15.5 cm. H_2O. Duration of greatest resistance, 145 min. Lung-heart ratio, 2.12.

Weight of animal, 16.5 kilos. Total barbital-sodium, 0.33 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.05 sq. cm. Negative intratracheal pressure, 15 cm. H_2O. Duration of greatest resistance, 70 min. Lung-heart ratio, 1.53.

than the shade of a normal lung. Hypostatic congestion in Experiments 3 and 4 was moderate. The lungs in Experiment 2 were completely free of hypostasis.

The results of these experiments may be summed up as follows: Resistance to inspiration results in a fall in intratracheal pressure which is associated with an increase in respiratory rate and a decrease in tidal air. In most instances these are accompanied by a severe limitation of the minute volume of pulmonary ventilation. The effects come on suddenly when the cross-section area of the inspiratory passageway is reduced to 0.1 sq. cm. or 0.05 sq. cm. Anoxemia accompanies these changes, but may be prevented by the inhalation of 90 to 95 per cent oxygen. Associated with the anoxemia there is a retention of carbon dioxide and usually a drop in pH.

When resistance is removed the respiratory rate continues to be rapid. In the majority of instances the rates were higher following release than they were during the period of resistance. Release, however, permits an increase in tidal air and minute volume, and as a result of this, there is a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia. On only one occasion when resistance was removed did the respiratory rate return to the control level, with a corresponding change in CO_2 tension and oxygen saturation. The postmortem picture found in these dogs may be characterized by congestion and edema of the lungs.

II. Experiments with Resistance in the Expiratory Phase.

In striking contrast to the experiments with resistance in the inspiratory phase, partial obstruction to expiration slows the respiratory rate. This occurs with varying effects on tidal air, but with a constant decrease in minute volume of pulmonary ventilation. The results of four experiments are presented in Tables V to VII.

In Experiment 11 (Table V) the dog breathed at the rate of 25 before resistance was introduced in expiration. In the presence of restriction equal to 0.1 sq. cm. the rate dropped to 12 and the tidal air increased from 85 cc. to 122 cc. This was accompanied by a decrease in minute volume from 2.12 liters to 1.46 liters with only slight changes in pH and blood gases. The animal exhaled against resistance for 96 minutes. 24 minutes after the tap was opened the

respiratory rate had returned to the original level—thus distinguishing this type of experiment from the ones in which resistance was introduced into inspiration. No other conspicuous changes occurred. The data are given in Table V.

In Experiment 12 (Table VI) a drop in the respiratory rate from 22 to 11 was accompanied by a decrease in tidal air from 70 cc. to 49

TABLE V.

Experiment 11. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	$\frac{mm}{Hg}$		*mM*	*mM*	*per cent*
Control period	12.08 to 12.39	Room air	25	85	2.12	26.62	45.15	7.37	6.35	6.68	95.1
During resistance	12.39 to 2.30	Room air	12	122	1.46	25.87	40.23	7.41	6.69	7.15	93.6
After release	2.30 to 3.12	Room air	24	110	2.64	24.51	35.72	7.44	6.24	6.59	94.7

Weight of animal, 10.5 kilos.
Total barbital-sodium, 0.32 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Positive intratracheal pressure, 13 cm. H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1.51.

cc. and a decrease in minute volume from 1.56 liters to 0.48 liter. The animal breathed room air, and, as could be anticipated from the great reduction in pulmonary ventilation, developed a severe anoxemia. The CO_2 content of the serum rose from 29.13 mM to 32.48 mM, the pCO_2 from 51.1 mm. Hg to 70.0 mm., and the pH dropped from 7.36 to 7.26. The period of resistance was 96 minutes. When the resistance was removed the respiratory rate returned to within five

breaths of the control level. Coincident with the recovery in rate there was an increase in tidal air and minute volume, with the result that the anoxemia was completely relieved and the pCO_2 and pH returned to their previous values.

In Experiments 1B and 10 (Table VII) there was likewise a noticeable decrease in respiratory rate and minute volume.

TABLE VI.

Experiment 12. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mм*	*mm. Hg*		*mм*	*mм*	*per cent*
Control period	12.40 to 1.14	Room air	22	70	1.56	29.13	51.1	7.36	7.01	7.53	93.1
During resistance	1.14 to 2.50	Room air	11	49	0.48	32.48	70.00	7.26	4.41	8.18	53.9
After release	2.50 to 3.31	Room air	17	84	1.42	28.71	48.23	7.38	7.43	7.70	96.5

Weight of animal, 11.5 kilos.
Total barbital-sodium, 0.30 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Positive intratracheal pressure, 12 cm. H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1.14.

The postmortem examinations in this group of experiments brought out the following points: None of the animals showed pleural effusions, frothy fluid in the trachea, edema of the mediastinal tissues, or gross edema of the lungs. The color of the lung surface in all instances was a diffuse coral-pink, with the same slight variations in intensity as were noted in the previous series. Perhaps the most striking observation was a complete absence of hypostatic congestion in Ex-

TABLE VII.

Experiments 1B and 10. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Experiment 1B					Experiment 10				
	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume
				cc.	*liters*				*cc.*	*liters*
Control period	3.15 to 3.43	90–95 per cent O_2	12	166	2.03	1.40 to 2.22	Room air	16	162	2.53
During resistance	3.43 to 6.07	90–95 per cent O_2	9	184	1.59	2.22 to 4.10	Room air	9	138	1.24
After release	6.07 to 6.26	90–95 per cent O_2	8	200	1.68		Room air	—*	—	—

Weight of animal, 13.5 kilos. Total barbital-sodium, 0.32 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.1 sq. cm. Positive intratracheal pressure not recorded. Duration of greatest resistance, 20 min. Lung-heart ratio, not determined.

Weight of animal, 11 kilos. Total barbital-sodium, 0.37 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.1 sq. cm. Positive intratracheal pressure, 10 cm. H_2O. Duration of greatest resistance, 74 min. Lung-heart ratio, 1.04. * The resistance was not released in this experiment.

periments 1B and 12, with only a slight degree of hypostasis in Experiments 10 and 11. The lung-heart ratios, calculated in three out of the four instances (Experiments 10, 11, and 12) were 1.04, 1.51, and 1.14, respectively. All of these figures are lower than those

obtained with resistance in the inspiratory phase, though at times the difference is very slight.

These experiments may be summarized thus: Resistance to expiration slows the respiratory rate and limits the minute volume of pulmonary ventilation. These changes may or may not be accompanied by a retention of carbon dioxide and a low oxygen saturation of the arterial blood. The effects come on suddenly when the cross-section area of the tap is reduced to 0.1 sq. cm. With removal of resistance all functions return to their normal, or approximately normal, levels. No characteristic pulmonary pathology occurs as the result of resistance to expiration.

DISCUSSION.

The second group of experiments is easier of interpretation than the first. With resistance to expiration there is a mechanical limitation to pulmonary ventilation which may result in carbon dioxide retention and insufficient oxygenation of the blood in the lungs. When the mechanical limitation is removed both the breathing and the state of the blood return to normal and no apparent damage to the lungs has occurred.

The interpretation of the first group of experiments, those dealing with resistance to inspiration, is not so clear. Here, too, we have a mechanical limitation to pulmonary ventilation, but the result in this case is rapid and shallow breathing rather than slow and shallow. There is, as in the other group, inadequate ventilation of the blood. But, even after the resistance had been removed, in many experiments the respiratory rate continued to be rapid, and in some this was true even though the blood had returned to the state found during the control period.

What has happened to the animal which causes it to maintain a rapid respiratory rate in spite of the fact that no longer is there any resistance to the free passage of air into the lungs? The explanation must be sought for in alterations to one of the organ systems having to do with respiratory rhythm. Has the respiratory center itself been damaged or fatigued? There does not appear to be any direct evidence for this assumption. The more or less prolonged period of anoxemia and acidosis which existed in the inspiratory ex-

periments was present in one of the expiratory experiments as well, and yet in this animal there was no accelerated rate after release of resistance. Have the muscles which have to do with inspiration become fatigued as the result of resistance? This hardly seems to be a plausible explanation because in none of the experiments was there a fall in the negative pressure produced in the trachea. Such a fall would have suggested a lessened effort at expansion of the lungs.

The explanation may be sought for more probably in the state of the lungs themselves. The decreased expansion may give rise to a state of pulmonary congestion and this, together with the heightened negative pressure, may result in a seepage of fluid into the pulmonary parenchyma and pleural sacs, as has been suggested by Graham (10). Indeed, it has been experimentally shown by Huggett (11) that inspiratory obstruction increases the minute and stroke volumes of the heart, while expiratory obstruction produces a reverse effect. An augmented blood flow through the lungs may be responsible for the congestion and fluid transudation which were actually observed. It was not unlike that seen in dogs with multiple experimental emboli of the pulmonary capillaries and arterioles (2), nor unlike the changes found after clamping and releasing the artery to one lung (4). In these conditions, too, persistent rapid and shallow breathing occurred.

The local changes in the lung may then perhaps be regarded as responsible for this phenomenon. The normal Hering-Breuer stimuli are increased and predominate. Whether this in itself is evidence of fatigue of the respiratory center, as Haldane believes, is a matter for conjecture.

SUMMARY AND CONCLUSIONS.

1. A study has been made of the effects of resistance to respiration in the inspiratory and expiratory phases.

2. Resistance to inspiration caused an increase in respiratory rate, a decrease in tidal air, and in most instances a severe limitation of the minute volume of pulmonary ventilation. Anoxemia and acidosis accompanied these changes.

3. When resistance was removed the respiratory rate continued to be rapid, but the tidal air and minute volume increased. As a result of this there was a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia.

3. Resistance to expiration slowed the respiratory rate and produced a constant decrease in the minute volume of pulmonary ventilation. Anoxemia and carbon dioxide retention occurred, but were less pronounced than in the inspiratory experiments. Release of resistance to expiration resulted in a return of all functions to their normal, or approximately normal, levels.

4. A difference in the gross pulmonary pathology found at autopsy in these two types of experiments has been described, and an attempt has been made to correlate changes in function with changes in structure.

5. No direct evidence has been supplied for the liability to fatigue of the respiratory center.

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A FURTHER STUDY OF BLOOD REACTION AND BLOOD GASES IN PNEUMONIA.

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Three years ago two of us with Neill and Morgan (1) attempted to determine whether or not a condition of acidosis exists in pneumonia. The hydrogen ion concentration, carbon dioxide content and tension, and the oxygen content and capacity were estimated in the blood of sixteen patients. In all, thirty observations were made. No tendency towards an acidosis of either metabolic or respiratory origin was observed. Indeed, the alkali reserve was within normal limits in every case. The pH, too, was in each instance within normal limits, 7.30 to 7.50, the greater number of observations falling in the more alkaline half of this range. The results, as are those of this study, were believed to contraindicate alkali therapy.

In spite of the consistency of the findings in this first series, it was thought advisable to extend the observations to a larger number of patients, and particularly to make repeated successive estimations on the blood of a few individuals. A patient suffering from pneumonia may present such a rapid succession of changes with respect to his hematorespiratory function, that he can scarcely be said to be in a steady state. What is true of him one day may not be true of him the next.

This fact, together with the changes induced by such environmental factors as serum therapy and oxygen inhalation, naturally gives one pause in generalizing about the state of the blood in so inconstant a condition as pneumonia. The results of the present study are, however, corroborative of the earlier one.

Material and Methods.

The observations reported were made on adult patients of both sexes, all suffering from pneumonia. Nineteen individuals comprise the series on whom

TABLE I.

Observation No.	History No.	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO₂ content per liter (mm)	CO₂ tension (mm Hg)	O₂ content per liter (mm)	O₂ capacity per liter (mm)	Saturation (per cent)	Temperature (°C.)	Pulse	Respiration	Breathing room air	Breathing O₂, O₂ in chamber (per cent)	Treatment	Remarks
1	5230	2- 2-25, a.m.	Type I	R. U. L.	6			7.30	21.52	52.4	6.02	8.18	73.6	101.4	118	40			Type I serum	Empyema Surgical drainage Suppurative pleurisy and pericarditis Death
2		2- 2-25, p.m.		R. U. L.	6			7.48	21.20	36.0	7.75	8.51	91.1	100.4	102	40		39	Type I serum	
3		2- 5-25, a.m.		Empyema	9			7.45	25.80	44.8	6.74	6.93	97.3	100.4	98	40		33	Type I serum	
4		2- 5-25, p.m.		Empyema	9			7.50	25.22	39.1	5.73	6.95	82.5	101.4	96	36	Yes			
5		2- 6-25		Empyema	10		42	7.47	24.52	40.7	5.95	7.01	84.9	101.4	104	36	Yes			
6	5233	2- 4-25	Grp. IV	R. U. L.	3	6		7.53	22.03	33.1	7.59	7.80	97.3	100.4	125	24	Yes		Transferred for operation	Recovery
7		2- 6-25		R. U. L.	5			7.38	24.92	51.9	7.30	8.51	85.9	100.4	88	32	Yes			
8	5253	2-20-25	Type I	R. U. L.	3		4	7.42	22.45	45.3	8.13	9.36	86.8	101	120	32		38	Type I serum	Bilateral sero-fibrinous pleurisy. Death

Grp.	No.	Case	Date	Organism	Lobe	Day		pH									Type I antipneumococcus serum		Remarks
4	9	5257	2-21-25, a.m.	Grp. IV Staph.	R.L.L.	2	3	7.47	22.38			6.65	68.3	100.4	120	40	Yes		Bilateral serofibrinous pleurisy. Fibrinous pericarditis. Death
	10		p.m.			2		7.50	22.56	37.6	7.42	9.69	76.6	100.4	130	36		38	Death
5	11	5259	2-23-25	Grp. IV	L.L.L.	2		7.45	19.95	36.3	6.44	8.51	75.7	103.4	130	36	Yes		Recovery
	12		2-24-25		L.L.L.	3											Yes		
6	13	5268	3- 2-25	Grp. IV	R.U.L.	4	7	7.46	22.96	38.9	5.73	6.82	84.0	102.1	76	31	Yes	39	
	14		3- 3-25			5		7.49	24.20	38.3	6.01	6.53	92.0	101.6	72	30		41	
	15		3- 4-25			6		7.49	24.86	39.1	6.15	6.32	97.3	101	80	34		39	
	16		3- 5-25			7		7.40	26.70	50.2	5.90	6.40	92.2	98.6	64	28			
	17		3- 6-25			8		7.48	25.60	40.1	5.70	6.01	94.9	97.6	60	22	Yes		
	18		3- 7-25			9		7.44	24.28	41.9	5.94	6.52	91.1	97.8	68	24	Yes		
	19		3- 9-25			11		7.34	19.69	42.1	6.31	6.58	95.9	98.4	68	22	Yes		
	20		3-10-25			12		7.45	20.27	34.8	6.32	6.86	92.2	98.6	60	25	Yes		
	21		3-13-25			15		7.37	21.74	43.6	6.40	6.40	96.4	98.2	76	20	Yes		
7	22	5278	3- 8-25	Grp. IV	R.L.L.	4	11	7.41	23.03	43.9	6.31	6.93	91.1	103	108	28	Yes		Recovery
	23		3-10-25			6		7.44	23.31	41.4	6.28	7.16	87.7	101.3	108	24	Yes		
	24		3-11-25			7		7.46	23.58	41.7	6.29	6.71	93.8	100.4	88	32	Yes		
8	25	5292	3-19-25	Grp. IV	L.L.L.	3		7.40	20.59	41.5	7.56	7.95	95.1	103.6	104	28	Yes		Recovery
9	26	5300	3-23-25	Type I	L.L.L.	4	9	7.37	25.60	53.4	4.92	7.67	64.2	102	110	48	Yes	40	Empyema. Surgical drainage. Recovery
	27		3-24-25		R.U.L.	5		7.33	26.82	60.9	7.15	7.63	93.7	101	92	36			
	28		3-25-25			6		7.43	23.73	43.7	6.68	7.83	85.4	100.4	72	34	Yes		

TABLE I—Concluded.

Case No.	Observation No.	History No.	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO₂ content per liter (mM)	CO₂ tension (mm. Hg)	O₂ content per liter (mM)	O₂ capacity per liter (mM)	Saturation (per cent)	Temperature (°C.)	Pulse	Respiration	Breathing room air	Breathing O₂ in chamber (per cent)	Treatment	Remarks
10	29	5310	3–27–25	Type I	R. M. L. R. L. L.	5		10	7.38	24.30	52.7	7.56	9.76	77.5	103.2	106	32	Yes		Type I anti-pneumococcus serum	Death
	30		3–28–25			6			7.49	23.41	39.8	7.69	8.84	87.0	104	118	50		39		
	31		3–31–25			9			7.42	29.61	52.1	3.55	4.98	71.3	101.8	138	56		38		
11	32	5314	3–30–25, a.m.	Type III	L. L. L.	7	13		7.33	20.70	44.8	3.55	5.68	62.5	102.8	86	32	Yes			Recovery
	33		3–30–25, p.m.						7.49	21.80	33.3	4.26	5.33	80.0	102	84	50		50		
12	34	5317	4–6–25	Staph. aureus	R. L. L. R. M. L.	7			7.50	21.55	32.1	4.88	5.37	90.9	101.2	100	28		40		Empyema Surgical drainage. Recovery
13	35	5347	5–18–25, a.m.	Type III	L. U. L. L. L. L.	7	7		7.34	21.95	50.1	6.47	9.07	71.3	100.6	124	32	Yes			Septicemia (Type III). Chronic morphine poisoning. Recovery
	36		5–18–25, p.m.							23.55		8.80	8.97	98.1	98.2	112	32		50		
	37		5–23–25			12			7.48	27.40	45.5	6.19	8.02	77.2	99.2	104	36	Yes			
	38		5–25–25			14			7.41	28.44	54.2	7.20	7.64	94.3	98.8	76	22		40		
	39		6–2–25			15				27.20		5.24	7.96	65.8	100.4	90	28	Yes			
	40		5–26–25			22			7.44	32.55	6.25	5.53	6.43	86.0	98.6	73	16	Yes			

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			Date	Type	Lobe						Yes?	Notes	Outcome
14	41	5470	12-15-25	Type III	L. L. L.	4	5	7.47	25.88	41.8	5.74	96.5	102.2	100	32	Yes		Recovery
15	42	5472	12-21-25	Grp. IV	R. U. L. R. L. L.	7	12	7.38	25.76	48.6	2.74	4.41	62.1	102	120	42	Yes		Recovery
16	43	5476	12-19-25	?	L. L. L.	5	11	7.37	4.99	5.22	5.6	104	72	25	Yes		Recovery
17	44	5490	12-30-25, a.m.	Type I	L. U. L. I. L. L.	4		7.50	32.62	49.13.02	5.48	55.1	102	130	38	Yes		Recovery
45			12-30-25, p.m.				12	7.46	4.65	5.29	87.9	102.3	116	32	45		Type I serum. Transferred for operation.	Empyema. Surgical drainage. Recovery
18	46	5509	1-12-26	Grp. IV	L. U. L. L. L. L.	3	7	7.40	22.58	43.9	6.13	7.18	85.4	102.4	116	30	Yes		Confluent bronchopneumonia. Death
19	47	5522	2-1-26	Grp. IV	R. U. L. R. M. L. R. L. L.	12	13	7.36	32.22	65.6	3.70	6.32	58.6	99.4	120	70	40			Death

R. U. L. signifies right upper lobe; R. M. L., right middle lobe; R. L. L., right lower lobe; L. U. L., left upper lobe; L. L. L., left lower lobe.

forty-seven observations were made. The number of observations per individual varied from one to as many as nine. Blood for analysis was drawn by puncture, usually of the femoral artery. The customary precautions were taken against exposure of the blood to air. Analysis of the total carbon dioxide content, oxygen content and capacity, was performed by the method of Van Slyke and Neill (2). The hydrogen ion concentration was measured by the colorimetric method of Hastings and Sendroy (3).

Calculations.

The data presented in Table I and graphically plotted in Fig. 1 were derived, as in the first paper, by calculation from the analyses of
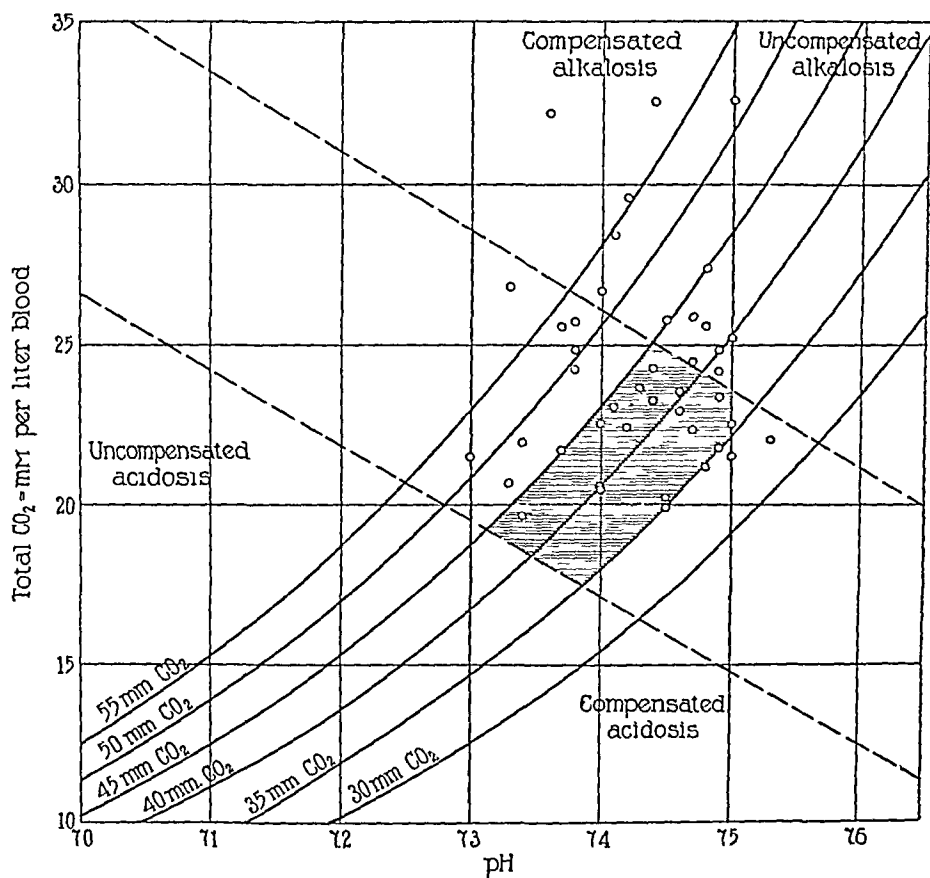
FIG. 1.

pH of serum, and CO_2 content, oxygen content, and oxygen capacity of whole arterial blood.

The fundamental equation used was the familiar Henderson-Hasselbalch formula for whole blood:

$$(1) \qquad pH_{t^\circ} = pK_1'_{t^\circ} + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

$$= pK_1'_{bt^\circ} + \log \frac{[CO_2] - [H_2CO_3]}{[H_2CO_3]}$$

where t° = body temperature in °C., $pK_1'_{bt^\circ}$ = the negative logarithm of the apparent first dissociation constant of carbonic acid in whole blood at body temperature, and the brackets indicate concentrations in terms of mM per liter whole blood.

$$(2) \qquad [H_2CO_3] = \frac{[CO_2]}{1 + 10^{\,pH - pK_1'_{t^\circ}}}$$

The pH of serum, as usual, was used for whole blood. The value of $pK_1'_b$ was calculated from the equation of Van Slyke, Wu, and McLean (4).

$$(3) \qquad pK_1'_b = pK_1'_s + \Delta pK_1'$$

where $pK_1'_s$ stands for pK_1' in serum, and $\Delta pK_1'$ is the correction applied for the difference in pK_1' in whole blood from the serum value. From the pH, oxygen content, and oxygen saturation, $\Delta pK_1'$ was estimated by using the nomogram of Van Slyke, Hastings, Murray, and Sendroy (5). To estimate $pK_1'_{bt^\circ}$, the data of Stadie and Martin (6) were used to calculate the temperature coefficient of this constant. Their values give $\frac{\Delta pK_1'_b}{\Delta t^\circ} = -0.0065$. Hence the complete expression for $pK_1'_{bt^\circ}$ is:

$$(4) \qquad pK_1'_{bt^\circ} = pK_1'_{s38^\circ} + \Delta pK_1' \pm \Delta t^\circ \,(-0.0065)$$

where $pK_1'_s = 6.12$, and $\pm t^\circ$ is the difference in °C. between body temperature at the time of bleeding and 38°C.

To estimate the CO_2 tension, the expression:

$$(5) \qquad [H_2CO_3] = \frac{\alpha CO_{2bt^\circ} \times pCO_2}{760 \times 0.0224} = 0.0587 \alpha CO_{2bt^\circ} \times pCO_2$$

was used, where αCO_{2bt° is the solubility coefficient of CO_2 in whole blood at body temperature, and pCO_2 is the CO_2 tension. The value of αCO_{2bt° being propor-

tional to the water content of the blood. this quantity was calculated as follows. Using equation 30 of Van Slyke, Wu, and McLean (4),

$$(6) \qquad (H_2O)_b = 0.914 - 0.015 \; (Hb)$$

where $(H_2O)_b$ = kilos H_2O per kilo blood, and Hb = mM Hb per kilo blood as measured by oxygen capacity determinations, the expression:

$$(7) \qquad H_2O_b = \frac{0.914 \; (Gb) - 0.015 \; Hb}{1.007}$$

was derived, where H_2O_b = liters H_2O per liter blood, (Gb) = specific gravity of blood, and Hb = mM per liter blood. (Gb) was calculated from equation 29 of Van Slyke et al.:

$$(8) \qquad (Gb) = 1.027 - 0.0037 \; Hb$$

According to Bohr and Bock, αCO_{2H_2O} at $38° = 0.555$ and the temperature coefficient at this temperature is -0.0124.

$$(9) \qquad \text{Hence } \alpha CO_{2_{bt°}} = [0.555 \pm \Delta t° \; (- \; 0.0124)] \; H_2O_b$$

By combining all of the previous equations, the complete expression for the CO_2 tension is found to be:

$$(10) \quad pCO_2 = \frac{[CO_2]}{0.0583 \times [0.555 \pm \Delta t° \; (- \; 0.0124)] \times [0.939 - 0.01162 \; Hb] \times \left[1 + 10^{pH \, - \, [6.12 \, + \, \Delta pK_1' \, \pm \, \Delta t° \, (- \, 0.0065)]}\right]}$$

Due to the fact that the average Hb concentration and degree of saturation was different from that of the group of cases reported in the first paper, the results, when plotted on the diagram of Hastings, Neill, Morgan, and Binger (1), showed a CO_2 tension different from that calculated. Hence a new diagram was drawn based on calculations of a blood having an average oxygen capacity of 7 mM Hb per liter, and an oxygen saturation of 82 per cent. Calculated CO_2 tensions in most instances agree with those plotted in Fig. 1.

RESULTS.

The results are tabulated in Tables I and II and graphically plotted in Fig. 1, on the modified Van Slyke acid-base diagram.

The pH of the Serum.—Of the 45 observations but one pH was higher than 7.50, all the rest falling within the normal limits of 7.30 to 7.50. The average pH of this series is 7.43. Table II shows a higher frequency of pH on the alkaline side of this normal range.

TABLE II.

Showing the Frequency of Incidence of the Variables Calculated in Table I.

pH................	7.30–35	7.36–40	7.41–45	7.46–50	7.51–55			
Incidence........	5	11	11	17	1			
CO_2, *mm per liter*....	19–21	21.01–22.0	22.01–24.0	24.01–26.0	26.01–28.0	28.01–30.0	30.01–32.0	32.01–34.0
Incidence........	5	5	12	14	4	2	0	3
pCO_2, *mm*....	30–35	35.1–40.0	40.1–45.0	45.1–50.0	50.1–55.0	55.1–60.0	60.1–65.0	65.1–70.0
Incidence........	4	8	14	4	9	1	1	1
O_2 saturation, *per cent*....	75 and below	76–80	81–85	86–90	91–95	96–100		
Incidence........	12	4	5	7	13	6		

The CO_2 Content of the Blood.—The average of 42 measurements gave a value of 24.26 millimols per liter, which is well within the normal range.   Most of our results were clustered about this figure for CO_2 content.

The CO_2 Tension.—Of the 42 CO_2 tensions calculated, half were within the normal limits of 35 to 45 mm., most of the remainder occurring above the higher of these values.   The average was 44.4 mm.

The Oxygen Saturation.—The average value for oxygen content was 6.02 mM per liter, and for oxygen capacity 7.12 mM per liter.   The average percentage saturation was 84.5 per cent, irrespective of oxygen therapy.

These results, in so far as one may make any inferences under such varying conditions of the disease and its treatment, corroborate, in every respect but one, the observations of the previous paper on this subject.   This series fails to show the correlation previously noted, between the CO_2 tension and the patient's temperature.   None of the points plotted in Fig. 1 fall in an acidosis area.   In fact, those points not in the normal area tend to indicate a condition of compensated alkalosis.   The CO_2 content showed little departure from normal values, as has already been noted by various other investigators.   The CO_2 tensions, on the other hand, were grouped more in the region of the higher normal values.   The oxygen unsaturation in these observations was more marked than that of the first series.

SUMMARY AND CONCLUSIONS.

Estimation of the blood reaction and of blood gases in a series of nineteen individuals suffering from pneumonia failed to reveal a condition of acidosis occurring at any time during the disease.   The results of this study are corroborative of a previous one, to which reference has been made.

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STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

II. The Infectivity of Type III Pneumococcus for Rabbits.

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In a previous paper (1) it was shown, in agreement with the experience of others, that the sera of rabbits immunized with Type III pneumococci failed to agglutinate the homologous organisms. Evidence was offered that the failure in agglutination was due, not to the inagglutinable state of the encapsulated bacteria, but to the actual absence of demonstrable type-specific antibodies. However, the sera of the immunized animals were reactive with pneumococcus nucleoprotein regardless of its type derivation and were capable of agglutinating the non-encapsulated degraded variants of all type-specific strains (R forms). This antibody response is contrary to the usual experience in immunization with type-specific pneumococci, when the cells used as antigen are intact. Although the Type III organisms used were, for the most part, possessed of large capsules, the antibody response, instead of being type-specific, was only species-specific in character and simulated that elicited by immunization with pneumococcus protein or with non-encapsulated R forms. The inference drawn from these results was that rabbits possess some mechanism which is capable of affecting the antigenic integrity of Type III pneumococci and that the alteration which the organisms undergo in the animal body is reflected in the character of the antibody response.

Because of the unusual reaction of rabbits to immunization with Type III pneumococci and the implications which these results suggest, investigations have been carried out with regard to the infectivity of this type of pneumococcus for rabbits. The experiments reported in the present paper include observations on the degree of viru-

lence of several strains of Type III, on the character of the bacteremia following intravenous injection, and the relation of phagocytosis to the disposal of the injected organisms.

Although Type III pneumococci are recognized as being highly pathogenic for white mice, reports on their virulence for rabbits have differed. Hanes (2) states that Type III organisms are highly virulent for rabbits without giving dosage or number of strains tested. Singer and Adler (3) found 0.05 to 0.1 cc. of culture usually fatal, but mention the fact that the lethal dose, in their experience, was not constant. They employed two strains and found one more constantly virulent than the other. Lévy-Bruhl (4) found the minimum lethal dose to be 1 cc. with two strains of Type III and greater than 1 cc. with two other strains. Bengtson (5) reported the lethal dose to vary from 0.1 to 1 cc.; whether different strains were tested or not is not stated.

In order to obtain further information concerning the virulence of Type III pneumococci for rabbits, eleven strains of this organism were collected. The strains were obtained from either blood or sputum of patients suffering from lobar pneumonia. All of the strains, which had been recently isolated were found on first injection to be highly virulent for mice. Other strains, which were taken from stock, were first passed through mice to enhance their virulence for these animals before being tested in rabbits. This was done in order to exclude from the cultures degraded R forms of pneumococci, which are non-encapsulated, non-type-specific, and avirulent. Reimann (6) has pointed out that a pneumococcus culture of low virulence may be one in which R forms predominate over type-specific, encapsulated S forms. According to this view, the repeated passage through mice of a culture containing both forms increases the proportion of S organisms until, with the acquisition of maximum virulence, the culture is theoretically composed entirely of type-specific, encapsulated pneumococci. Consequently, the cultures of Type III used for virulence tests in rabbits, by first being made highly pathogenic for mice, fulfilled this requirement.

Rabbits were injected either intravenously or intraperitoneally, with 12 to 14 hour cultures in doses ranging from 2 to 10 cc. It may be seen from Table I that eight of the strains did not produce fatal infection. In the case of two other strains rabbits died following injection of relatively large doses, but the cultures isolated from the

blood of these failed to kill others. Although no sustained effort was made to enhance the virulence of all the strains, most of them were injected several times. Of the eleven strains only one acquired definite virulence for rabbits, which, by repeated passage, finally produced a fatal infection in doses of 0.0001 cc. Even with this strain

TABLE I.

Virulence of Type III Pneumococcus for Rabbits.

Strain of Type III	Virulence for mice	Rabbit No.	Dose of culture	Site of injection	Results
	cc.		*cc.*		
A......................	.000001	1	5	Intravenous	Survived
A......................	.000001	2	2	Intraperitoneal	"
A......................	.000001	3	2	"	Died—2 days
Blood culture Rabbit 3..		4	2	"	Survived
" " "		5	5	"	"
M......................	.000001	6	2	Intravenous	"
M......................	.000001	7	3	Intraperitoneal	"
M......................	.000001	8	10	"	"
F......................	.000001	9	5	"	Died—3 days
Blood culture Rabbit 9...		10	5	"	Survived
F......................	.000001	11	8	"	"
L......................	.000001	12	2	Intravenous	"
L......................	.000001	13	5	Intraperitoneal	"
H......................	.000001	14	5	Intravenous	"
H......................	.000001	15	10	Intraperitoneal	"
E......................	.000001	16	5	"	"
S......................	.000001	17	5	"	"
S......................	.000001	18	8	"	"
B3.....................	.000001	19	5	"	"
B3.....................	.000001	20	2	Intravenous	"
B4.....................	.000001	21	5	"	"
B4.....................	.000001	22	5	Intraperitoneal	"
B2.....................	.000001	23	5	"	"
PH*....................	.000001	24	5	"	Died—2 days

* This strain became highly virulent for rabbits.

the degree of virulence has not remained constant and has often shown evidence of attenuation when kept out of the animal body for several days. Moreover, differences in the natural resistance of individual rabbits to the same strain is, in part, responsible for variations in the degree of virulence.

From the results obtained it seems justifiable to conclude that Type III pneumococci possess only slight initial virulence for rabbits. Furthermore, although type specificity and encapsulation are necessary for the highest degree of mouse virulence, these characteristics are not, in themselves, sufficient to overcome the natural resistance possessed by rabbits.

The conclusion that Type III pneumococci are of low pathogenicity for rabbits was arrived at by the simple procedure of injecting organisms and accepting the ultimate survival or death of the animal as a criterion of virulence. This method, however, throws no light either on the duration and intensity of the infection, or upon the method of recovery, a fact which has been emphasized by Bull (7) in a report on some of the characteristics of streptococcal and pneumococcal bacteremia in rabbits. The technique employed by him consisted in making blood cultures intermittently from the peripheral veins after the injection of organisms. The results obtained by Bull and others (7–10) have demonstrated the reliability of the method. Consequently, since Type III pneumococci failed to produce fatal infections in rabbits, it seemed of interest to observe the course of the bacteremia following injection of these organisms. This was done by means of blood cultures taken at frequent intervals following the introduction of organisms into the circulation. The results are diagrammatically represented in the accompanying text-figures in which the number of colonies per unit of blood is plotted on the ordinates and the time interval, at which the culture was taken, is plotted on the abscissæ (Text-figs. 1, 2, and 3).

There were available for this study both S and R strains of Type III pneumococci. The eleven S strains were typical and biologically identical. They possessed large, easily demonstrable capsules; they grew on blood agar with the production of mucoid colonies; they were bile-soluble; they all reacted equally well in Type III antipneumococcus horse serum. They were pathogenic for humans, the source from which they were derived, and were all equally virulent for mice, killing in doses of 0.000001 cc. However, one of the S strains differed in that it was made virulent for rabbits by rabbit passage, whereas the others were not virulent for these animals in doses ranging from 2 to 10 cc. In addition to the type-specific S strains of pneumococci, non-

type-specific, degraded R forms were used for comparative study. As described by Reimann (6) R forms may be obtained by cultivating type-specific organisms in homologous immune serum. Those used in this experiment were derived from a culture of Type III. Doses of 1 cc. failed to kill mice and doses of 10 cc. failed to kill rabbits; higher doses were not tested. The strains of pneumococci used in the present experiment, then, comprised representatives of each of the three varieties:

1. S strains of Type III pneumococcus; *virulent* for rabbits (designated SV). One strain belonged to this group.

2. S strains of Type III pneumococcus; *avirulent* for rabbits (designated SA). Ten strains belonged to this group.

3. R strains representing the degraded, non-encapsulated, avirulent variants of Type III pneumococci. Bacteria of this character are comparable to non-pathogenic saprophytes.

EXPERIMENTAL.

Cultures.—12 to 14 hour plain broth cultures in standard doses of 2 cc. each were injected intravenously regardless of the strain used. The actual number of organisms per cc. was not determined but under uniform conditions, it may be considered comparable for all the strains.

Blood Culture Technique.—The technique employed varied only in minor details from that described by Bull (7) and was as follows: The organisms were introduced into the marginal vein of one ear of the rabbit and cultures were obtained from the opposite ear. Before making the culture, the ear was closely shaved along the marginal vein and then saturated with 95 per cent alcohol. The alcohol was wiped off with a dry sterile sponge and the vein slit transversely with a razor blade. The blood was allowed to drip perpendicularly from the edge of the ear and, after discarding the first few drops, 6 drops were collected in melted agar kept at 42° to 45°C. The melted agar and blood were mixed rapidly and poured into a Petri dish. The plates were incubated for 36 hours and the number of colonies per plate recorded. Cultures were usually taken 15, 30, and 60 minutes after injection of the organisms, then at 2 hour intervals for 12 hours; after this, 2 or 3 times daily until the animal succumbed or permanent sterility occurred. After the first 12 hours subsequent cultures were taken from blood derived from a fresh slit distal to the previous cuts. This precaution was taken in order to obviate the possibility of organisms resident in the tissues at the site of the previous injury being washed into the agar by the flowing blood.

Employing streptococci and pneumococci Bull (7) found that infection in rabbits may take one of three courses depending on the viru-

lence of the bacteria.   With highly virulent organisms a rapidly fatal septicemia occurred which was characterized by an initial marked reduction in the number of organisms in the circulating blood, followed by a rapid increase.   With less virulent organisms the infection became chronic, the course of the bacteremia was uneven, and, after death, localization of organisms in the serous cavities was often demonstrable.   When bacteria of even lower virulence were injected, they quickly disappeared and the blood stream remained sterile.   The initial diminution in the number of organisms occurring in experimental septicemia, first described by von Fodor (8), has been repeatedly observed by others.   This phenomenon has usually been explained as being due to the dissemination and filtration of the organisms throughout the body tissues.   However, that this explanation is not sufficient becomes evident, as will be shown, if the relative virulence of the bacteria injected is taken into consideration.

Course of Bacteremia Following Intravenous Injection of Rabbit Virulent, S Strain of Type III Pneumococcus (SV Strain).

The effect of increased virulence on the course of the bacteremia is shown in Text-fig. 1.   These results were obtained by injecting rabbits with a strain of Type III pneumococcus, the virulence of which had been progressively increased by repeated passages through rabbits.   The data presented reveal differences in the course of the bacteremia depending on the degree of virulence.   When the minimal lethal dose of the rabbit virulent strain (SV) was 0.5 cc. there was a marked initial decrease in the number of organisms in the circulating blood, the minimum occurring 4 hours after infection; then there followed a progressive rise in the number of colonies until the death of the animal occurred 56 hours after injection.   This type of curve in experimental septicemia corresponds to the results previously described by others.   When the virulence of this same strain had been enhanced so that now, 0.01 cc. of culture proved fatal, the initial decrease was less marked, and the secondary rise more rapid.   Injection of a culture of still greater virulence (0.001 cc.) resulted in only a slight initial decrease in the number of organisms in the peripheral blood.   When a maximum virulence of 0.0001 cc. had been attained the number of organisms in the blood of the infected rabbit was at no

time decreased and death resulted in 12 hours. In each instance approximately the same number of organisms was injected. Therefore, if the primary decrease represented merely a mechanical process of dissemination and filtration it should occur regularly, regardless of virulence. This, however, was not the case; the extent of the initial decrease in the number of circulating bacteria was in inverse proportion to the degree of virulence (Text-fig. 1).
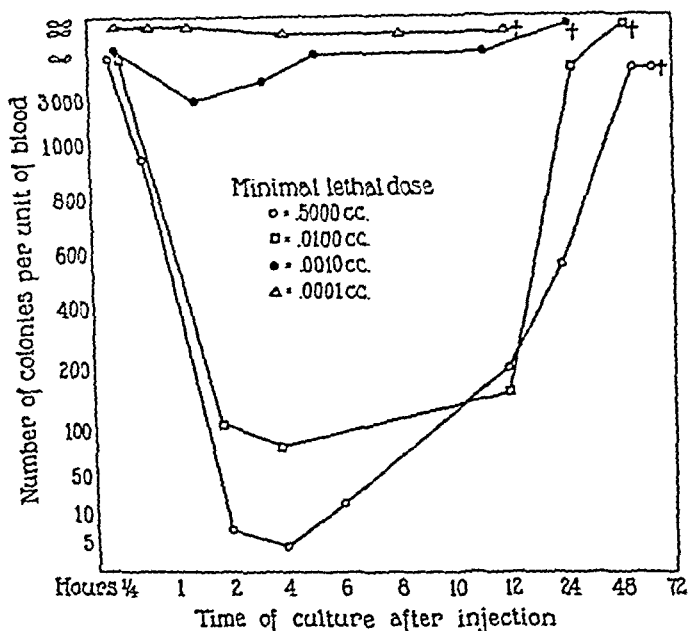
TEXT-FIG. 1. Effect of increased virulence on the course of bacteremia in rabbits injected with Pneumococcus Type III (Strain SV).

In addition to the effect of progressively increased virulence on the course of blood infection caused by the rabbit virulent strain of pneumococcus, certain characteristic differences in the curves were observed when strains of Type III not virulent for rabbits and degraded R forms of pneumococcus were employed. A graphic representation of the course of the bacteremia following injection of organisms of these two varieties is shown in Text-fig. 2. For purposes of comparison the type of curve representing the course of events after injection of the rabbit virulent strain is included in this same experiment.

Course of Bacteremia Following Intravenous Injection of R Strains of Pneumococcus.

From Text-fig. 2 it may be seen that there was an immediate and marked decrease in the number of R organisms in the circulating blood. After 2 hours they completely disappeared and the rabbit remained free from further blood infection.
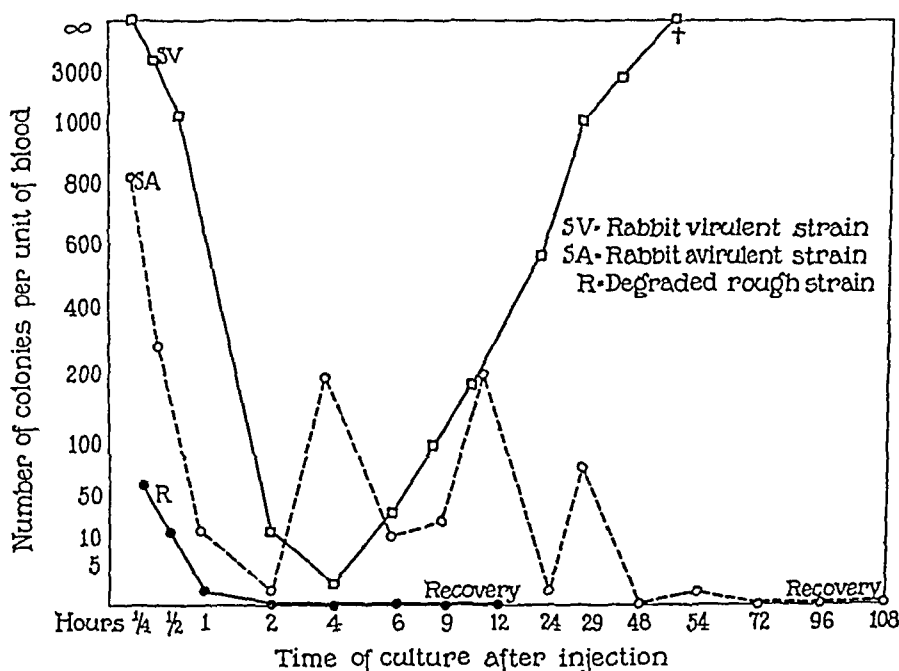
TEXT-FIG. 2. Course of bacteremia in rabbits injected with Pneumococcus Type III (Strains SV, SA, R).

Course of Bacteremia Following Intravenous Injection of Rabbit Avirulent, S Strains of Type III Pneumococcus (SA Strains).

The bacteremia which followed injection of rabbit avirulent strains of Type III pneumococci ran a distinctly different course. From Text-fig. 2 it may be seen that 3 to 4 days elapsed before permanent sterility of the blood was finally attained. While the bacteremia persisted, the chief characteristic was repeated fluctuations in the number of organisms in the circulating blood. At times the cocci entirely disappeared only to reappear again a few hours later. Exami-

nation of the organisms which reappeared failed to reveal any biological differences, when compared with the original cultures. As previously stated, the rabbit avirulent strains (SA) represent all but one of those used in the experiments. Although only one curve characteristic of the bacteremia produced by these strains is shown in Text-fig. 2, repetitions of this experiment gave similar results. In Text-fig. 3 are shown similar curves obtained with three other rabbit avirulent strains.
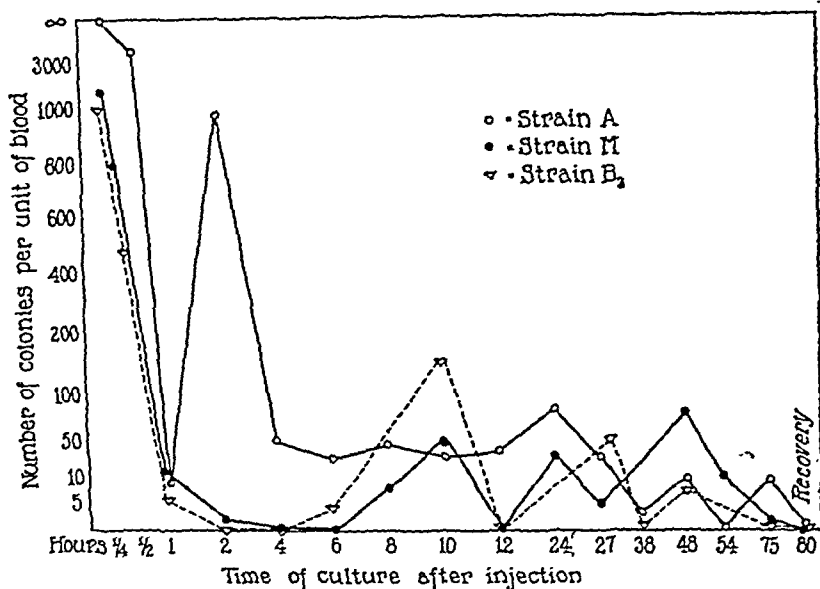
TEXT-FIG. 3. Course of bacteremia in rabbits injected with strains of Pneumococcus Type III, avirulent for rabbits (Strains SA).

In the experiments just described virulence of Type III pneumococci for rabbits has been considered with special reference to certain properties pertaining to the organisms themselves, such as differences of encapsulation, type specificity, and mouse virulence. These factors have been correlated with the infectivity of the organisms as represented, not only by the ultimate outcome of the infection in rabbits, but also by the character of the bacteremia produced.

In addition to the biological properties of the bacteria, factors related to the resistance of the host require consideration and an investigation of two of these has been carried out.

1. Antibodies.—The sera of normal rabbits have not been found to possess antibodies reactive with the encapsulated Type III cells, or with the soluble specific substance derived from these organisms, or to confer passive protection on mice. Although normal rabbit sera are occasionally encountered which agglutinate R pneumococci, the incidence is not common and the dilutions of serum in which the reaction occurs are low. Whether antibodies of this character are significant in the disposal of R cells after injection into the animal body, is irrelevant to the present study. It can be stated, however, that the phagocytosis of R pneumococci, as described below, is not preceded by agglutination.

2. Phagocytosis.—In studying phagocytosis, the vital staining method described by Sabin (11) for the study of living blood cells has been employed. The technique varied in no detail from Sabin's procedure except for the fact that a small loopful of living pneumococcus culture was added to the blood. The preparations were observed microscopically in a warm chamber kept at 37°C. By this method leucocytes may be watched immediately after removal from the animal body and while actively motile in the environment of whole blood. Preparations of this character approximate conditions existing in the circulating blood and minimize alterations of the leucocytes which may occur in the usual methods of studying phagocytosis *in vitro.* The specimens were observed from 1 to 2 hours. At the end of this time the leucocytes begin to lose their motility and their protoplasm contains large, red staining bodies which Sabin has described as vacuoles. Observations beyond 1 to 2 hours have not revealed phagocytosis which was not present earlier, and the beginning alteration in the leucocytes was evidence that the preparations were not useful for further observation.

When R forms of pneumococci were employed, phagocytosis by polymorphonuclear leucocytes could be seen in 2 to 3 minutes. It continued actively and, after 10 minutes, every leucocyte was engorged with organisms. When phagocytosis is observed under these conditions of vital staining the picture is very striking. Pneumococci, before ingestion, are unstained. However, immediately after being engulfed, they appear as bright red organisms within the body of the leucocyte. The facility with which R cells are phagocyted is

evidence of the prime importance of this activity in the natural resistance of rabbits to blood infection with R forms; the promptness with which these organisms disappear from the blood stream following intravenous injection may be referable, in part at least, to their susceptibility to phagocytosis.

When S forms of Type III pneumococci were mixed with normal rabbit blood, no phagocytosis was observed. This was true whether the encapsulated organisms were virulent or avirulent for rabbits. Occasionally, after an hour a few leucocytes could be found which had ingested one or two bacteria but the picture contrasted sharply with that seen in preparations made with R forms. Since the inability of leucocytes to ingest encapsulated bacteria has been a common observation, and, since this fact has been related to virulence, the failure to demonstrate this correlation in the present study is of special significance. Although phagocytosis as observed *in vitro* may not be identical with the phenomenon *in vivo*, nevertheless, the contrast between the action of leucocytes against R and S forms is striking and justifies the inference that the natural resistance of rabbits to S forms of Type III pneumococci involves factors either additional to or different from phagocytosis.

DISCUSSION.

In the experiments reported in this paper on the infectivity of Type III pneumococci for rabbits certain factors which are known to be associated with virulence and resistance have been taken into consideration. The organisms used in the experiments conformed with certain requirements which might be expected to promote virulence. All of the strains possessed large mucoid capsules and type specificity (S forms). They were obtained from patients suffering from lobar pneumonia and were highly virulent for mice. However, in spite of this, ten out of eleven of these strains failed to produce fatal infection in rabbits when injected in moderately large doses. The other strain was slightly virulent for rabbits on isolation and this property was further enhanced by animal passage. Since no biological differences could be demonstrated between this strain, when possessed of maximum virulence, and the others, it is necessary to assume that viru-

lence in this instance is related to some property not possessed by the other S strains. The course of the fatal septicemia resulting from injection of this strain and alterations in the character of the curve associated with increased virulence are shown in Text-fig. 1.

An attempt to understand the failure of *avirulent, encapsulated* Type III pneumococci (S forms) to cause fatal infection in rabbits led to a study of the fate of these organisms as contrasted with that of *avirulent, non-encapsulated* pneumococci (R forms). Although the ultimate survival of the infected animal occurs in both instances, the bacteremia induced by S forms differs from that induced by R forms. Following injection, the non-encapsulated R cells disappear rapidly and permanently from the blood stream, whereas the avirulent, but encapsulated S forms give rise to a prolonged bacteremia characterized by intermittent increase and reduction in the number of circulating organisms. These differences suggest that the mechanism whereby rabbits overcome the two types of infection is not identical, and that the method of disposal is in some way related to the presence or absence of capsule. Further evidence that the mechanism of recovery in the case of S organisms is different from that effective in disposing of R forms, was brought out in the experiments on phagocytosis. Therefore, it seems obvious that, although the encapsulated state is sufficient to prevent phagocytosis, it is not sufficient to protect the cell against the defense reactions of the host. The resistance of rabbits to Type III pneumococci implies the presence of factors other than phagocytosis. Furthermore, the fact that the sera of normal rabbits do not contain demonstrable type-specific antibodies renders improbable the participation of these immune substances in the mechanism of natural resistance. In a previous paper (1) it was shown that immunization of rabbits with Type III pneumococci failed, in the majority of instances, to stimulate the production of type-specific antibodies, but was effective in producing antibodies reactive against another constituent of pneumococci, namely the nucleoprotein fraction. These experiments indicate that normal rabbits possess a mechanism which is capable of inflicting an injury on the capsule of Type III pneumococci. It has been shown by Avery and Heidelberger (12) that type-specific antibodies are best elicited when S cells in an intact state are used as antigen. They (12) have also

shown that type specificity is intimately related to the soluble specific substance of the capsule of pneumococcus. Therefore, the absence of demonstrable type-specific antibodies in rabbits immunized with encapsulated Type III pneumococci indicates that the animals are capable of damaging the capsular mechanism of these organisms. In the present experiments, the capacity of normal rabbits to inflict injury on Type III pneumococci is further demonstrated by the recovery of the animals following intravenous injection of living S organisms. It seems not unlikely that both the survival of the animal and the altered antigenicity of the cell following injection of the encapsulated organisms are referable to the same mechanism, and upon the factors underlying this defense reaction depends the natural resistance of rabbits to Type III pneumococci.

SUMMARY.

The observations recorded in this paper on the infectivity of Type III pneumococci for rabbits may be summarized as follows:

1. Of eleven strains of Type III isolated from human sources, ten were found to possess low virulence for rabbits. This was true despite the fact that all the strains tested possessed large capsules and a high degree of virulence for mice.

2. One strain of Type III pneumococcus was rendered highly virulent for rabbits. Since it possessed no other biological property demonstrably different from the other strains, its virulence must reside in some additional property.

3. An initial decrease in the number of circulating organisms following the injection of virulent bacteria is a well known occurrence, and it was observed in rabbits injected with the rabbit virulent strain of Type III. However, the extent of the reduction was in inverse proportion to the degree of virulence of the strain; a fact which makes mechanical explanations of the phenomenon insufficient.

4. The bacteremia produced in rabbits by Type III pneumococci, avirulent for this species, runs a characteristic course. It differs from that produced by non-encapsulated R forms of pneumococci although in both instances survival of the infected animal ensues. This is evidence that the mechanism of resistance against encapsulated and non-encapsulated pneumococci is not identical.

5. Phagocytosis of Type III pneumococci by circulating rabbit leucocytes was not demonstrable by a vital stain technique, whereas under the same conditions the ingestion of non-encapsulated R forms occurred. This is further evidence that the process whereby non-encapsulated pneumococci are disposed of, is insufficient to explain the natural resistance of rabbits to infection with encapsulated Type III pneumococci.

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